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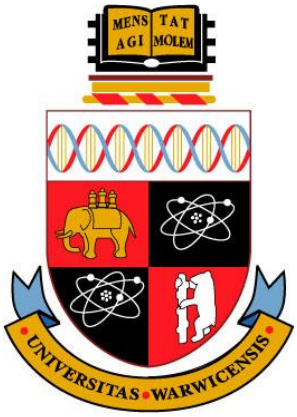

THE UNIVERSITY OF WARWICK

CHARACTERISATION OF HLA-SPECIFIC ANTIBODIES

David Philip Lowe

Thesis

Submitted to the University of Warwick

For the degree of

Doctor of Philosophy

Warwick Medical School

March 2013

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And finally, and most importantly, thanks Adele. You never complained when I didn't come home on time! Maybe now I'll have a little more time for you and our boys.

Declaration

I declare that all the work presented in this thesis, except where specifically stated, was original research performed by myself under the supervision of Dr Daniel Zehnder, Dr Daniel Mitchell, and Professor David Briggs. None of this work has been previously submitted for any other degree. All sources have been acknowledged by means of references.

David Lowe

26th March 2013

Abbreviations

ABC Antibody binding capacity

AHG Anti-human globulin

AID Activation Induced Deaminase

AIT Antibody incompatible transplantation

ALG Anti-lymphocyte globulin

AMR Antibody Mediated Rejection

APC Antigen presenting cell

ATG Anti-thymocyte globulin

β_2 M β_2-microglobulin

BCA Bicinchoninic Acid

BCR B cell receptor

BSA Bovine serum albumin

CD Cluster of differentiation

CDC Complement dependant cytotoxic crossmatch

CDRs Complementarity determining regions

CNBr Cyanogen Bromide

CREGs Cross-reactive groups

CSR Class switch recombination

DCD Donors following cardiac death

DEA Diethylamine

DFPP Double-filtration plasmapheresis

DSA Donor Specific Antibody

DTT Dithiothreitol

EDTA Ethylene diaminetetra-acetic acid

eGFR Estimated glomerular filtration rate

ELISA Enzyme-linked immunosorbent assay

ESRD End-stage reanl disease

Fab Fragment for antibody binding

Fc Fragment crystallisable

FCS Foetal calf serum

FCXM Flow cytometry crossmatch

FFP Fresh frozen plasma

FITC Fluorescein isothiocyanate

GALT Gut-associated lymphoid tissues

GVHD Graft versus host disease

HDHE High dose hook effect

HLA Human Leukocyte Antigen

HLAi HLA Incompatible Transplantation

IFN-γ Interferon-gamma

Ig Immunoglobulin

IgA Immunoglobulin A

IgG Immunoglobulin G

IgM Immunoglobulin M

IgE Immunoglobulin E

IgD Immunoglobulin D

IL-2 Interleukin-2

IL-2R Interleukin-2 receptor

IL-4 Interleukin-4

IL-10 Interleukin-10

IPTG isopropyl-beta-D-thiogalactopyranoside

ITAM Intracellular tyrosine activation motif

KIR Killer cell immunoglobulin-like receptor

LB Luria broth

LDT Living donor transplantation

LPS Lipopolysaccharide

MAb Monoclonal antibody

MAC Membrane attack complex

MALT Mucosa-associated lymphoid tissue

MASP-1/2 Mannose-binding-lectin associated serine proteases

MBL Mannose binding lectin

MFI Mean fluorescence intensity

MHC Major Histocompatibility Complex

MPC Magnetic particle concentrator

MS Mass spectroscopy

MWCO Molecular weight cut-off

NIBSC The National Institute for Biological Standards and Controls

NK Natural Killer cells

PAMPs Pathogen associated molecular patterns

PBS Phosphate buffered saline

PE Phycoerythrin

PES Polyethersulfone

PP Plasmapheresis

RMF Relative median fluorescence

SABA Single antigen bead assays

SDS-PAGE Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SHARC Soluble HLA antibody removal column

sHLA Soluble HLA

sInh Soluble inhibition

SPR Surface plasmon resonance

Tc T-cytotoxic cells

TCR T-cell receptor

TdT Terminal deoxynucleotidyl transferase

Th T-helper cells

TMD Transmembrane domains

Treg T-regulatory cells

TPA Third Party Antibody

UHCW University Hospital of Coventry and Warwickshire

Abstract

A successful kidney transplant is the best treatment for established renal failure, yet around 300 patients per annum are denied transplants because they have antibodies, most notably directed against donor HLA or ABO in their blood, which have the potential to cause acute and chronic rejection of the transplant. Such antibodies are present in 25% (roughly 1750 of the 7000 on the kidney transplant waiting list) of the patients listed for a deceased donor transplant. Programmes to remove antibody and transplant patients across HLA antibody barriers have been developed, but are limited by a high rate of acute rejection.

This thesis explores the factors which may impact upon the pathogenicity of HLA-specific antibodies and also aims to enhance the understanding of the techniques used in the laboratory to define these antibodies. A range of studies were carried out examining factors such as the IgG subclass composition of the anti-HLA response. Assay variations were designed to enable a higher definition of antibody specificity to be achieved, and for the first time in the literature the direct quantification of HLA-specific antibodies in patient sera was performed.

In addition, proof of principle design and testing was carried out on a novel prototype therapeutic device for the selective depletion of HLA-specific antibodies directly from patient plasma and sera. The antibodies isolated from this approach were also used in studies to examine the factors which determine serum cytotoxicity.

Chapter 1

INTRODUCTION.

1.1 Renal Transplantation: Historical Perspective

The origins of renal transplantation can be traced back to the first decade of the 20th century and the pioneering work of Emerich Ullmann at the Vienna Medical School. In 1902 Ullmann performed the very first auto, allo, and xenotransplants using dogs and sheep [1], and later that same year attempted the first human kidney transplant when he grafted a pig kidney into a woman with end stage renal disease. Although unsuccessful these early experimental treatments led to advances in surgical techniques. Later that decade French surgeon Dr M Princeteau attempted further xenotransplant procedures, this time transplanting sections of rabbit kidney into children suffering from renal failure. These transplants were successful in producing a limited amount of urine for a few hours but ultimately failed within a few days leading surgeons to summise that 'unknown biochemical barriers' were present which prevented long-term kidney graft survival. In 1906 another French surgeon Dr Mathieu Jaboulay followed in the footsteps of Ullmann and performed a pig to human xenotransplant. Unfortunately, the 32 year female patient died within one hour of having the pig kidney connected via an artery in her arm [2].

In 1933 Russian surgeon Yurii Voronoy performed the first human to human kidney allograft using a cadaveric donor [3]. The recipient was a 26 year old female admitted to hospital with acute mercury poisoning as the result of a failed suicide attempt. Although the kidney showed initial good function and urine production graft function soon worsened and the patient died 21 hours after transplant. The reasons for graft failure were clear with both the prolonged warm ischaemia time of the kidney and an apparent blood group incompatibility between donor and recipient both causal factors. Analysis of the case notes made by Voronoy at the time show that he had considerable insight into the immunological characteristics of organ rejection [3].

Major advances in the understanding of transplant immunology came in the 1940s and 1950s through the pioneering work of Sir Peter Medawar. A zoologist by training, Medawar was performing research into tissue culture techniques at Oxford during the second world war when he was tasked by the Medical Research Council to investigate why a skin graft from one human being was not accepted as a permanent graft by the recipient [4]. In collaboration with Peter Gibson and then subsequently Leslie Brent, Medawar noted fundamental differences between auto and allografting of human skin both microscopically and clinically, and correctly attributed these to an immunological process [5-8]. Using rabbits as a test species Medawar described the time delay from grafting to rejection (first set rejection) and how that delay was reduced and the immune response amplified following a second graft from the same donor animal (figure 1.1A). He described invasion of the graft by lymphocytes and demonstrated experimentally that these lymphocytes, rather than the antibodies that they produced were responsible for the graft destruction (figure 1.1B).

These observations led to the recognition of the immunological processes that underpin the allograft response; 1) That the immune response is inducible by the introduction of the allograft. 2) That the immune response displays memory, as a repeat graft is rejected both more quickly and more vigorously. 3) The immune response displays specificity, as a new graft from a previously unseen donor is rejected following a reversion to first set rejection kinetics. In 1960 Medawar was awarded the Nobel prize for his work on immunological tolerance alongside Macfarlane Burnet who first postulated the hypothesis of acquired tolerance.

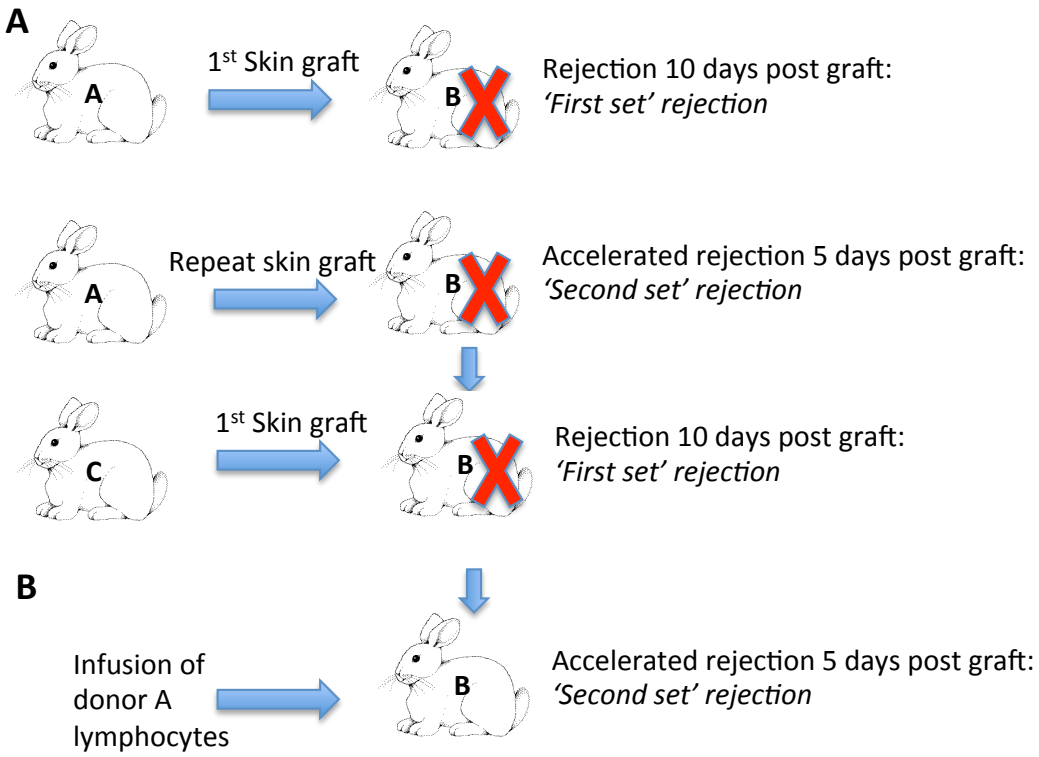


Figure 1.1: Principles of first and second set rejection demonstrated by the seminal experiments of Medawar [4]. A) A skin graft from strain A into B displays 10-day duration 'first set rejection', a repeat graft shows accelerated 'second set rejection'. Graft from another individual (Strain C) reverts back to first set rejection kinetics. B) Infusion of donor lymphocytes from strain A into a previously grafted strain B mouse results in second set rejection, highlighting the lymphocyte as the mediator of immunological memory.

Further advances were made in the 1950s, notably by Joseph E Murray at the Peter Bent Brigham Hospital in Boston USA who, in 1954, performed the first completely successful kidney transplant. The transplant involved a pair of monozygotic twin brothers and took place without any form of immunosuppression. Scientists correctly postulated at the time that immune reactivity between such individuals would

be minimal as their immune systems should be indistinguishable from each other. In 1956, Murray transplanted 20 year-old Edith Helm from her identical twin sister. At the time of her death in 2011 Edith had the longest surviving kidney transplant and was also the first person to safely give birth following renal transplantation [9].

The 1950s also saw early attempts at suppressing the recipient immune system in order to accommodate allografts in non-identical twin donor-recipient pairs. Early animal studies using cortisone and total body irradiation managed to prolong the survival of skin grafts but only by a matter of a few days [10, 11]. These findings focused the efforts of researchers into finding immunosuppressive drugs whose effects mimicked those of irradiation. The first reported case of extended graft survival using drug therapy alone was reported in 1963 [12] when Goodwin and colleagues in Los Angeles used methotrexate and cyclophosphamide to depress bone marrow activity and allow a mother to daughter kidney allograft to be performed. The kidney survived for 143 days with repeated rejection episodes associated with bone marrow recovery treated with prednisolone. Clinicians and surgeons soon realised that deliberate bone marrow depletion should be avoided due to the enhanced risk of infection, and studies performed by Schwartz and Dameschek demonstrated that 6-mercaptopurine (6-MP) could be used to induce immunosuppression at sub-myelotoxic doses [13]. Preclinical trials of 6-MP and its analogue azathioprine were carried out by Calne and Murray in Boston [14].

It is important to note that the advances described above took place at a time where there was little or no understanding of immunological screening or histocompatibility testing. The importance of the genetically determined major histocompatibility complex (MHC) in the immune response to allografts had been determined from studies using inbred mice strains performed by George Snell [15] inspired by earlier investigations from Peter Gorer [16]. It was only after the discovery

of the first human leukocyte antigen (HLA) in 1958 by Jean Dausset [17] and the subsequent discovery that very same year of anti-leukocyte antibodies (later shown to be directed against non-self HLA) by Van Rood [18], that the clinical utility of tissue matching and histocompatibility testing became a possibility. The description in 1964 by Terasaki and McClelland of the microcytotoxicity test allowed HLA antigens to be determined serologically using minute quantities of patient or donor sera [19, 20]. This in turn led to rapid advances in the classification of HLA antigens.

Despite these advances in tissue compatibility testing undoubtedly the most important advance in the pre-transplant testing regimen was the recognition that observing the guidelines of ABO compatibility and a negative pre-transplant cytotoxic crossmatch were the key points to avoiding hyperacute rejection. Hyperacute rejection as a result of ABO incompatibility was first reported in 1964, and strict guidelines were soon put in place to observe ABO barriers [21]. In 1965 the first reported case of hyperacute rejection in an ABO-compatible transplant was reported, with subsequent cases confirming that graft loss could be caused by pre-formed anti-graft lymphocytotoxic antibodies [22-24]. The weight of this evidence highlighting the effect of pre-formed cytotoxic antibodies on transplant survival led Terasaki to immediately recommend and implement the routine use of his lymphocytotoxic crossmatch test [25].

With the continued improvement in tissue matching that took place over the subsequent decades came the commonly held view that tissue matching would need to be perfected in order to sustain graft survival in the longer-term. This hypothesis would soon be shown to be true in the bone marrow transplant setting with even small degrees of HLA disparity between donor and recipient leading to graft versus host disease (GVHD) or graft failure, however these results were not replicated in kidney transplant studies [26, 27]. Although HLA matched grafts were shown to have the best survival and function, unexpectedly these studies showed there was no cumulative impact of

HLA mismatches on graft survival. This has been consistently shown in many transplant cohorts since with virtually all studies showing a statistical significance between different levels of HLA mismatching, however unless there is a near perfect HLA match between donor and recipient the impact of HLA matching is greatly diminished.

These advances in matching went hand in hand with important advances in immunosuppressive regimens throughout the 1970s, 80s and 90s. The importance of anti-lymphocyte globulin (ALG) was first demonstrated in the 1960s [28], given in conjunction with aziothioprine and prednisone, and as the name suggests ALG was a potent inhibitor of the lymphocyte response. The management of the dose of ALG was often difficult with under administration leading to potential allograft rejection and over-dosing leaving the patient liable to the severe side-effects of the drug; primarily infection and bone marrow disease. The development of the rosette inhibition test in the 1970s led to a more detailed clinical evaluation of ALG [29]. However, ALG suffered from batch to batch variability and this was not adequately addressed until the development of monoclonal antibodies [30]. OKT3 was a first generation monoclonal antibody specific for all T cell subsets used to treat rejection [31-33]. The discovery in the mid-1970s of cyclosporine would drastically improve the safety and reliability of solid organ transplantation. A potent suppressor of both humoral and cellular immunity, cyclosporine had a powerful and easily reversible effect on T cell activity. In animal studies cyclosporine did not depress the bone marrow and was less toxic to other organs, unlike aziothioprine [34]. When transferred to human trials, cyclosporine showed an ability to prevent rejection, either alone or in combination with other agents, far greater than any previously tested agent [35]. However, the large dosages required carried many serious side-effects, most notably nephrotoxicity. It was therefore necessary to combine lower dosages of cyclosporine with established agents such as

prednisone to taper these harmful effects. The prognosis of various solid organ transplant procedures was greatly improved as a consequence [36, 37].

Cyclosporine remained the mainstay of immunosuppression until 1989 when studies performed in liver transplant patients demonstrated that replacing cyclosporine with tacrolimus could rescue rejecting liver allografts [38], with further studies showing that tacrolimus could also rescue failing cardiac and renal allografts [39]. Harmful side-effects were seen to be comparable in magnitude to those associated with cyclosporine.

Further advances continue to be made in both the determination of histocompatibility and also in immunosuppression protocols. A more contemporary analysis will be given later in the context of antibody incompatible transplantation.

1.2 Renal Transplantation: The Growing Need

Due to the increasing success of renal transplantation and the great improvements in the safety of the procedure, the numbers of patients with end stage renal disease (ESRD) being put forward for transplantation each year far outstrips the number of cadaveric donors entering the donor pool. This problem is acutely felt in the UK, as the cadaveric donation rate is currently the lowest in Europe. The highest donation rate in Europe is seen in Spain, where three times the number of donors per head of population is observed compared to the UK. The reasons for this disparity are numerous and complex, with issues surrounding consent (Spain has a policy of assumed consent on organ donation), and age of potential donor involved.

Alternative strategies are increasingly being used to increase the potential donor pool and attempt to meet the demand for organs. The introduction of harvesting organs

from donors following cardiac death (DCD) has lead to a 300% increase in the use of this donor group in the last three years. For the financial year ending 31st March 2012 674 DCD transplants were carried out accounting for one in four of all kidney transplants performed in the UK in that period.

In addition, virtually all transplant centres have made concerted efforts to increase the rate of live donation, to the extent that in 2011-12, a total of 1009, over 1/3 of all transplants performed in the UK were taken from live donors, usually from close relatives of the patient. Despite these efforts data from the UK renal registry suggest that an additional 4000 kidney transplants need to be performed each year to meet demand, a 50% increase at least on current transplantation rates (figure 1.2).

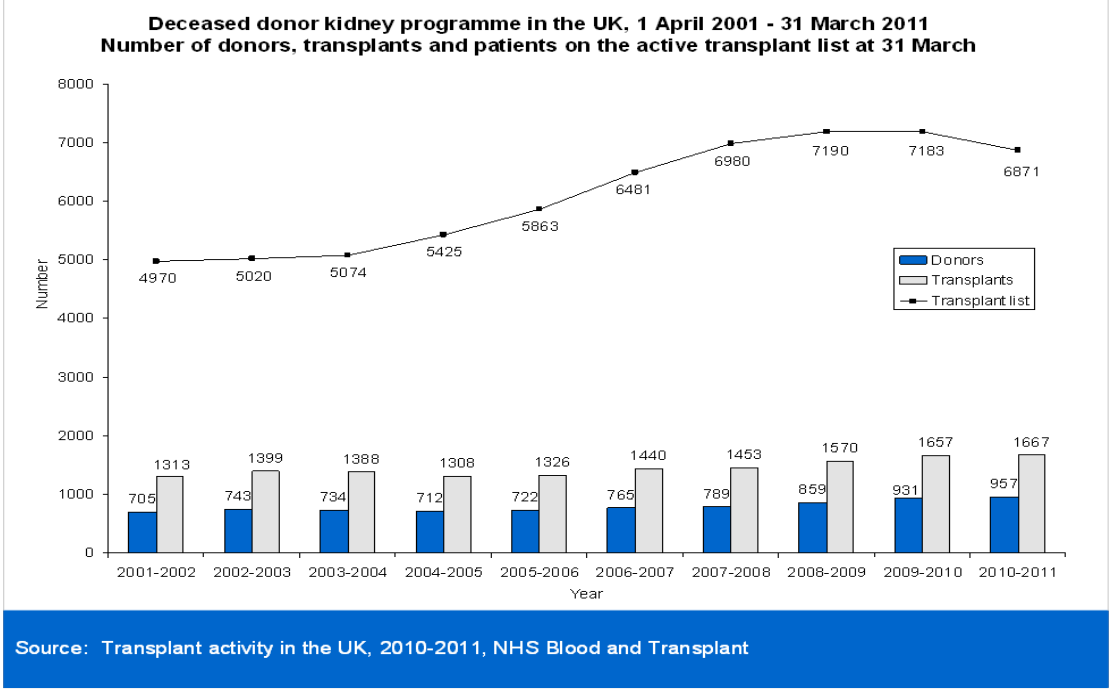


Figure 1.2: The number of deceased and living donors for the period 2001 to 2011.

Both the number of transplants performed and the number of available donors has remained fairly consistent during this period, however the number of patients awaiting transplant has for the most part increased (Source: NHS Blood and Transplant, Activity Report 2011).

There are also considerable cost savings to be made following renal transplantation. For example, from the second year of graft survival onwards there is an annual saving in the region of £25,000 per year in comparison to maintaining patients on dialysis. Therefore, UK transplant activity in the region of 2500 grafts per year delivers a cost saving in the region of £60 million per year of graft survival over the cost of maintaining patients on dialysis.

1.3 The Immune Response

The ability to recognize and respond to a bewildering array of potentially harmful foreign entities is the central task of the human immune system. Our immune system is a vast and complex interaction between many different cell types and molecules, the aim of which is to protect the host tissues from harm. The function of the immune system can be summarised into two key functions: 1) To recognise foreign organisms or substances that have entered the body and caused damage. 2) To remove the source of the tissue damage.

The immune response itself can be separated into two main areas, the innate immune response and the adaptive (acquired) immune response. Organisms that are able to breach our bodies' first-line physiological and anatomical barriers (skin, mucous secretions) will first encounter the innate immune system. This first-line immune defence response acts rapidly but is fairly indiscriminate, acting to destroy a wide range of pathogens. Acting in synergy with the innate response is the adaptive response which is much more sophisticated and specific for a particular antigen. As will be discussed in more detail in section 1.3.2 the adaptive response can also display immunological memory. However, this response takes much longer to develop and in reality many

initial immune responses will be comprised of a combination of both innate and adaptive elements (figure 1.3).

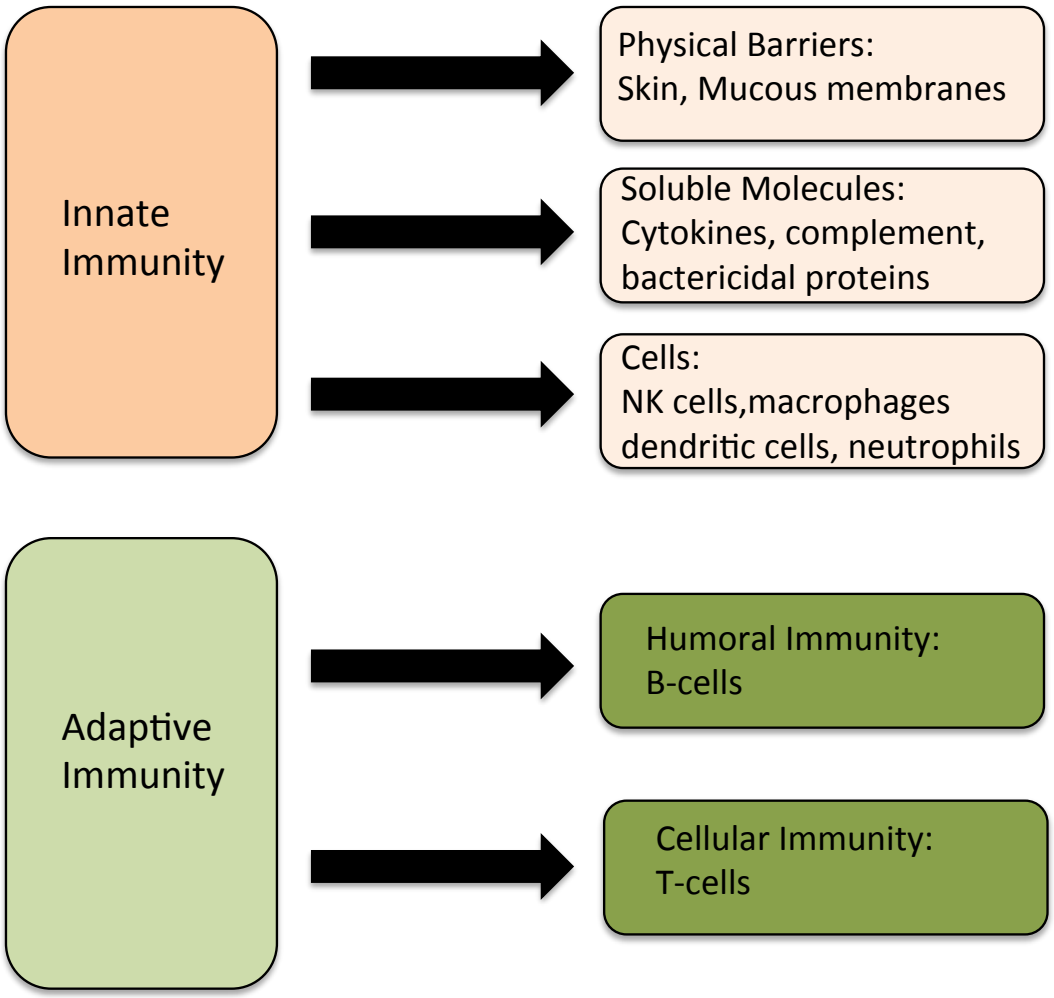


Figure 1.3: Overview of the Innate and Adaptive Immune Systems. The main elements of both the innate and adaptive arms of the human immune system. A detailed description of both is given in further details in section 1.3.1 and 1.3.2.

1.3.1 The Innate Immune Response

The innate immune response is a powerful first-line defence against host attack, so much so that in non-vertebrate species the innate response is sufficient for immune protection. A wide array of both haematopoietic and non-haematopoietic cells and molecules are integral to the innate response (table 1.1).

Table 1.1: Selected examples of components of the innate immune response to pathogen.

Component of Innate Immunity	Examples
Physical Barriers	The Skin Gut Villi Mucosal Surfaces Lung Cilia
Soluble Molecules	Cytokines Complement Proteins Bactericidal Lysozyme
Cells	Macrophages Neutrophils Natural Killer (NK) cells Dendritic Cells

The innate immune system is charged with trying to kill infectious particles the moment they are detected in the body, the actions of the innate immune response also alert the cells that operate the adaptive response. Importantly, although a powerful response the potency of the innate response does not improve upon repeat exposure to the same foreign agent. The innate response recognizes broadly conserved components of infectious antigens (sometimes referred to as pathogen associated molecular patterns, PAMPs) and is able to respond to these 'danger signals'. Although chemically distinct, these PAMPs have some shared features, notably that they are produced only by microbes and not by the host cells. In addition, they are usually essential for the survival

of these microorganisms and they are usually invariant structures shared by entire classes of microbes. For example, all gram-negative bacteria have lipopolysaccharide (LPS), therefore host cells containing receptors for LPS can recognise essentially all gram-negative bacteria. The constitutive expression of these pattern recognition receptors on cells such as macrophages, for example, allows a rapid response of the innate immune system to such infections. The innate response can directly engulf pathogens via phagocytosis or alternatively can attack them via the release of destructive enzymes such as proteases or lysozymes, with macrophages, neutrophils and components of the complement protein system being key players. This response occurs extremely rapidly, and indeed the innate response has to response this quickly when one considers that in the nutrient-rich environment of the human body pathogenic bacteria are often able to divide every twenty minutes [40].

1.3.2 The Adaptive Immune Response

Adaptive immune responses typically take around 4-5 days to develop and provide a more specialized and potent immune response mechanism. Often, innate immunity alone is not sufficient for pathogen clearance and assistance from adaptive immunity is required. Some examples of the key differences between innate and adaptive immunity are listed in table 1.2.

Most notably, adaptive immunity displays immunological memory and this is mediated by the two cell types which mediate adaptive immunity, the T- and B-lymphocytes. Arising from progenitor cells in the bone marrow, B-lymphocytes are responsible for antibody humoral immunity and T-lymphocytes and natural killer (NK) cells are responsible for cell-mediated immunity. Humoral immunity involves the

production of antibodies, molecules that can specifically recognize an almost infinite diversity of antigens and can also recruit various components of the immune response (this is discussed in more detail in section 1.5). Cell-mediated immunity comprises of cytotoxic T-cells (Tc) and the T-helper cell sub-groups (Th-1, Th-2). NK cells and dendritic cells also play a role in cell-mediated immune responses.

Table 1.2: Selected differences between the innate and adaptive arms of the immune response.

	Innate Immunity	Adaptive Immunity
Distribution	All living organisms	Vertebrates only
Specificity	Non-specific response	Antigen specific response
Response time	Immediate potent response	Time delay between exposure and response
Components	Physical Barriers Cells: Macrophages Neutrophils Dendritic cells Soluble proteins: Complement Lysozyme	 B cells (humoral immunity) T cells (antigen specific response)
Memory	No immunological memory	Immunological memory: Shortened lag-time and enhanced response to repeat exposure

Both T-cells and B-cells have surface receptors that recognize antigen. Each immature circulating lymphocyte expresses a prototype antigen receptor that is highly specific for a particular antigen. There are over one billion lymphocytes circulating in the human body, therefore there are only a very small number of circulating lymphocytes specific for each antigen that may be encountered. Lymphocytes that have

not yet encountered their cognate antigen are small, rather featureless cells termed naïve lymphocytes.

Upon encountering their specific antigen naïve T- and B-lymphocytes differentiate and become effector lymphocytes that are capable of eliciting immune responses via a number of different mechanisms. The way in which T- and B-lymphocytes encounter and recognize antigen is fundamentally different. B-lymphocytes are able to recognize antigen in soluble unbound form circulating in either the blood or the lymphatic system. T-cells, on the other hand require antigen to be presented to them specifically by antigen-presenting cells (typically dendritic cells or macrophages).

The cellular (T-cell mediated) adaptive immune response is initiated following an innate immune response whereby immature dendritic cells are able to engulf a pathogen by phagocytosis. This can initially be achieved in a receptor-independent manner or alternatively following recognition of bacterial LPS by cell surface receptors as discussed earlier. The immature dendritic cell then migrates to peripheral lymphoid tissue such as the lymph node, spleen, or mucosa-associated lymphoid tissue (MALT) where the pathogen is degraded into smaller pieces which are then processed and presented via the membrane bound major histocompatibility complex (MHC) molecule (a more detailed description of this process is given in section 1.4).

1.3.3 Clonal selection

As the human immune system can potentially produce hundreds of thousands, if not millions, of different T- and B-lymphocyte specificities, it is not practical to have large populations of lymphocytes recognising each specificity circulating at any given

time. Therefore the human immune system has developed to maintain a vast repertoire of potential antigen reactivity could be maintained but with far fewer circulating lymphocytes. Using the strategy of clonal selection (figure 1.4), lymphocytes that interact with their specific antigenic target, undergo successive waves of proliferation resulting in many more lymphocytes with identical antigenic specificity. In the case of B-cells each subsequent clone will become a plasma cell equivalent of the original parent lymphocyte capable of producing antibody molecules of exact specificity (a more detailed overview of the antibody molecule is given in section 1.5). T-cells will differentiate into various effector cells of either T-helper (Th), T-cytotoxic (Tc) or T-regulatory (Treg) type. Typically this process will take several days, hence the time-lag associated with adaptive immunity. Importantly however, a number of clones of both the T- and B-lymphocyte lineage will differentiate into memory cells capable of eliciting a greatly enhanced and potent response when repeat challenge with the same antigen occurs [41, 42]. This immunological memory is a key hallmark of the adaptive immune response and makes for a highly effective immune mechanism.

Any T lymphocytes which display self-reactivity (i.e makes a specific contact with a protein defined as self) are destroyed by apoptosis at a very early stage of development within the thymus. This is a process known as clonal deletion, which operates in tandem with clonal selection to maintain a non-self reactive pathogenic specific lymphocyte pool. This pool is capable of eliciting a rapid immune response when required.

Similarly, B lymphocytes are also tested for self-reactivity within the bone marrow. B cells found to display enhanced levels of self-reactivity can either be removed by apoptosis in a process known as clonal deletion [43], or alternatively clonal anergy can occur whereby B cells are maintained in a state of permanent unresponsiveness [44].

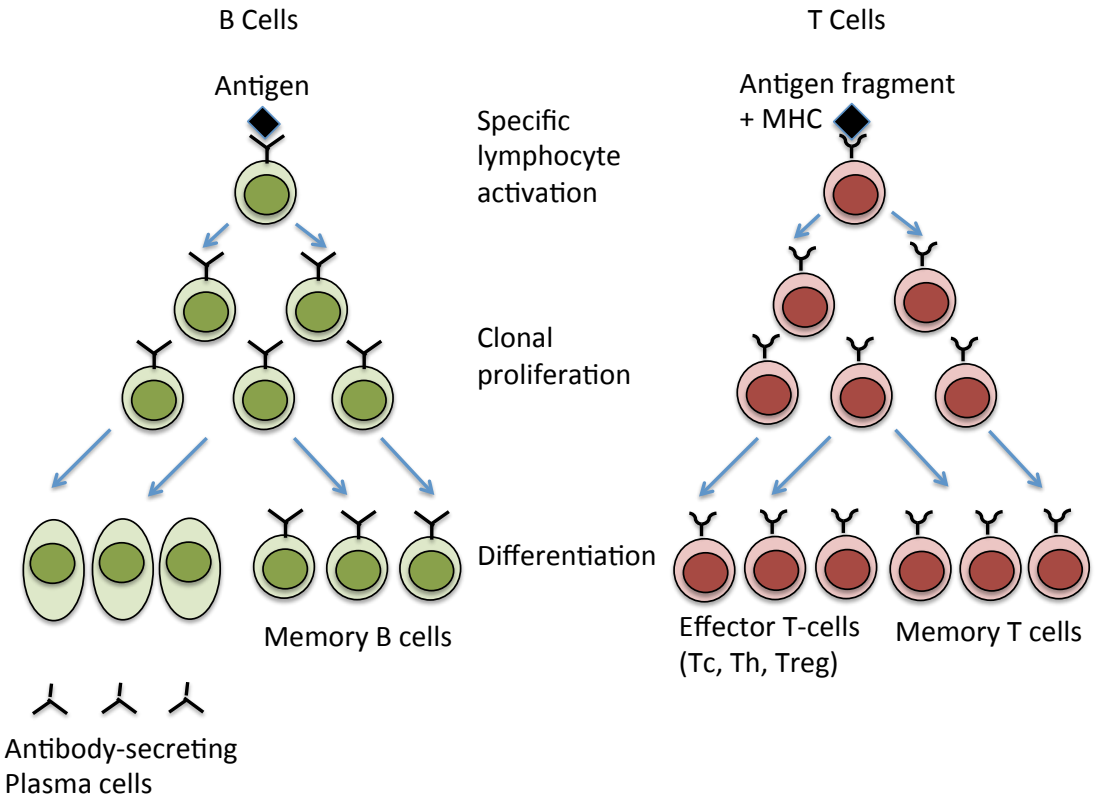


Figure 1.4: Clonal selection for the generation of effector and memory lymphocytes following antigen specific primary activation. Following specific activation of either a T- or B-lymphocyte a range of effector and memory cell populations can be propagated.

1.3.4 Cluster of Differentiation (CD)

The cluster of differentiation (sometimes referred to as the cluster of designation) is abbreviated to CD and serves as a nomenclature system used to identify cell surface molecules found on different leukocyte subsets. The CD nomenclature was first introduced at the 1st International Conference on Human Leukocyte Differentiation

Antigens which was held in Paris in 1982 [45]. The key benefit to this system of categorization was that it allowed monoclonal antibodies to be uniformally and unequivocally identified by the surface marker against which they are directed, regardless of the laboratory performing the research. A newly identified surface molecule is designated a unique CD designation once two or more separate monoclonal antibodies bind to the molecule. In the case where only one monoclonal antibody has been shown to be specific for a potential marker then the abbreviation CDw is used [45].

The CD nomenclature allows the definition of key cells involved in the immune response by virtue of the different combinations of markers found on their cell surface. The distribution of key CD antigens on important cells of the immune response are shown in figure 1.5. Many cells are defined primarily by virtue of the distribution of CD markers on their cell surface, and these markers are often used to associate particular cells with their function, e.g the CD8+ cytotoxic T-cell (refer to figure 1.5). Indeed it is important to remember that the CD molecules are more than just markers used to identify cell types but have important immunological functions, such as acting as ligands, receptors, or as cellular adhesion molecules. There are currently around 350 identified CD markers [46].

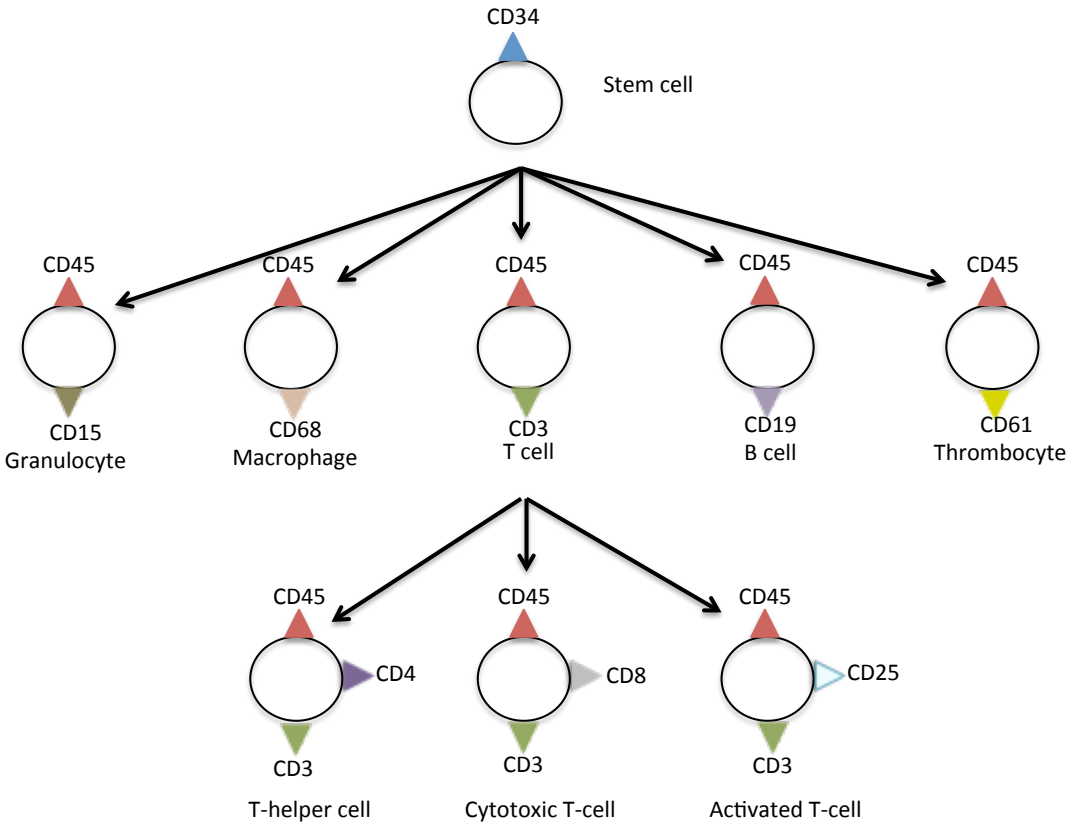


Figure 1.5: Cluster of differentiation (CD) markers for key cells involved in the immune response. Note all leukocytes are CD45+, and as a consequence CD45 is often dropped when referring to leukocyte subsets.

1.3.5 The T-cell receptor.

The T-cell receptor (TCR) is a molecule found on the surface of T cells and is responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. The TCR is a heterodimer composed of α and β chains, or less commonly, γ and δ chains. Each subunit chain of the TCR consists of a constant and variable region. The constant regions span the T-cell membrane, and the variable regions, which are distal to the T-cell membrane forms the antigen-binding region of the TCR (figure 1.6).

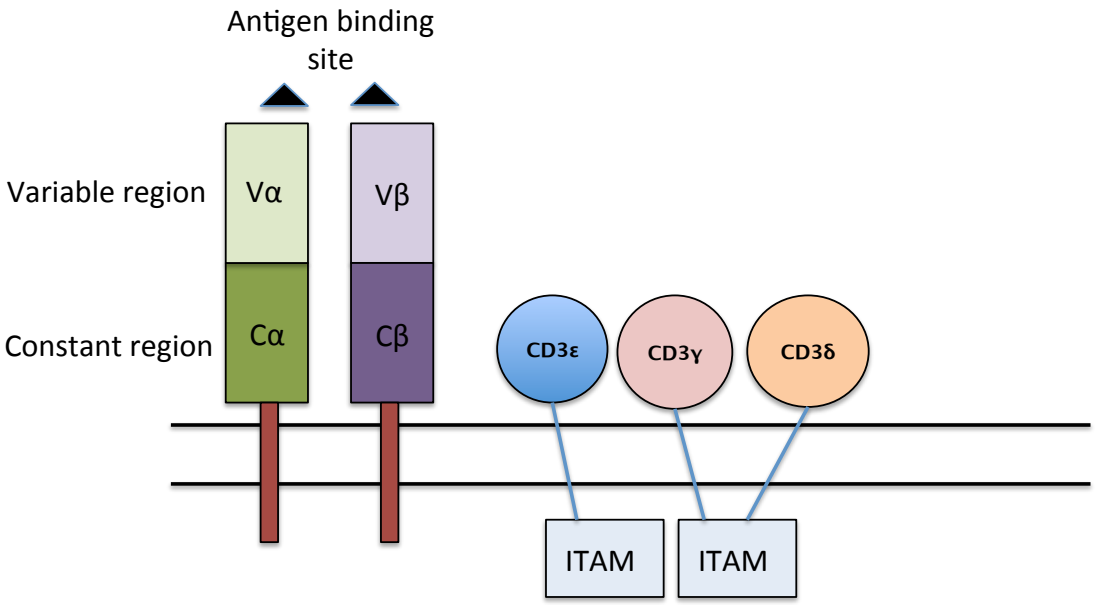


Figure 1.6: Simplified diagram of the TCR/CD3 complex. Upon binding of peptide/MHC complexes with the variable region of the TCR, a propagation of complex intracellular activation signals into the lymphocyte occurs which is mediated by the CD3 complex utilizing intracellular tyrosine activation motifs (ITAMS).

The TCR does not bind its antigen directly, it recognizes antigen only when presented by a class I or class II MHC molecule (sections 1.3.6 and 1.4). When the TCR does ligate antigen it relays a signal to activate the T-cell via an associated complex of transmembrane polypeptides, termed the CD3 complex (figure 1.6). The TCR is non-covalently linked to the CD3 complex, and it is the CD3 complex which activates the T-cells via phosphorylation of its attached intracellular tyrosine activation motifs (ITAM's). This signal transduction is assisted by the CD4/CD8 co-receptors, which also play a key role in antigen recognition involving the MHC/antigen complex.

1.3.6 The B cell

As outlined in figure 1.4, B cells can be stimulated to undergo proliferation (clonal expansion) and further differentiate into antibody secreting plasma cells, and long-lived memory B cells. The current understanding of how this process is performed and regulated is outlined below.

The B cell lineage contains a variety of cells with differing functions and a wide variety of surface (CD) markers. B1 cells, characterized by the surface marker CD5, are able to produce low-affinity 'natural antibody' independent of T-cell help, whereas as the B2 cells are involved more in the adaptive immune response. These B2 cells are initially formed in the bone marrow, and are then released as immature B cells whereupon they continuously circulate through secondary lymphoid organs such as the lymph node and spleen until they encounter antigen (figure 1.7). Upon activation, the B cell then interacts with its cognate T-helper cell [47]. This interaction involves the processing and presentation of antigen by the B cell, which then presents the antigen, complexed with the MHC class II molecule, for recognition by the TCR (figure 1.6).

A number of factors make B cells vitally important antigen presenting cells (APCs). Firstly, as outlined in figure 1.4, B cells have the ability to clonally expand and therefore rapidly amplify a specific immune response. Secondly, B cells are able to take up antigen extremely efficiently via their cell surface B cell receptor (BCR).

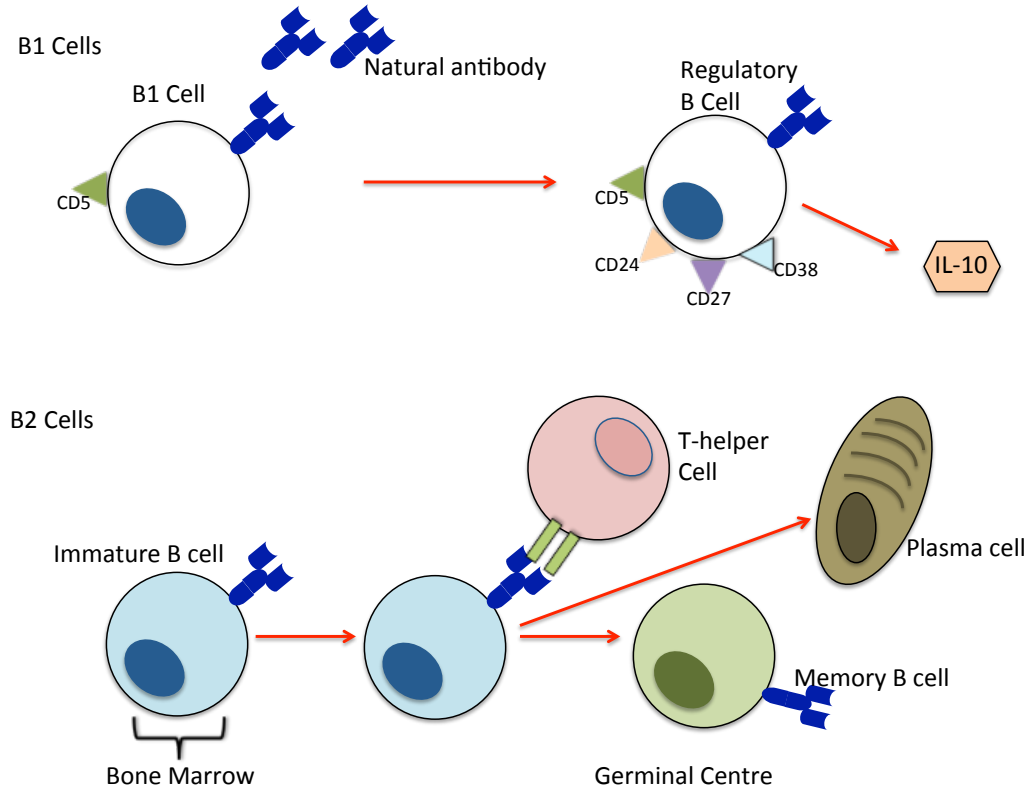


Figure 1.7: B cell differentiation. B1 cells are recently discovered B cells capable of producing low affinity natural antibody [47]. These B1 cells may differentiate into CD24+/27+/38+ regulatory B cells which play an important role in regulating T cell mediated responses. B2 cells are typically immature B cells produced in the bone marrow which migrate to the germinal centres where they encounter antigen, interact with T cells, then differentiate into either memory B cells or antibody producing plasma cells.

In addition, B cells are powerful mediators and regulators of specific T cell responses. B cells produce cytokines which act upon T cells, for example the activated B cell produces interferon-gamma (IFN-γ) which mediates T cell polarization and

differentiation into memory T-cells [48]. Regulatory B-cells, thought to derive from the B1 lineage and characterized by surface expression of markers CD24, CD27, and CD38 (figure 1.7), also inhibit T-cell specific responses primarily due to release of interleukin-10 (IL-10). These regulatory B-cells are thought to constitute around 5% of circulating B cells [49].

The fate of the activated B cells can follow one of two paths. They can either develop into extrafollicular plasmablasts that produce low affinity antibody, or they can enter the germinal centre where they undergo somatic hypermutation and class switch recombination (described in more detail in section 1.5). Within the germinal centre, B cells with higher affinity for antigen are positively selected and are then able to differentiate into either memory B cells or antibody producing plasma cells (figures 1.4 and 1.7). A small proportion of these B cells migrate to the bone marrow where they reside as long-lived plasma cells. These cells occupy a limited number of niche sites within the bone marrow and although they are unable to proliferate, they are able to act as long-term 'factories' constantly producing IgG [47]. The structure, function, and relevance to transplantation of the IgG molecule is discussed in detail in section 1.5.

The importance of the B cell in the immune response to transplantation is highlighted by the fact that a number of immunosuppressive strategies have been developed which target B cell functions and effector mechanisms. Direct B cell depletion can be achieved crudely via splenectomy, or more selectively via the administration of monoclonal antibodies which directly bind to markers expressed on the B cell surface. Anti-thymocyte globulin (ATG) and the anti-CD52 antibody alemtuzumab (Campath-1H) are both used to deplete B cell populations, although both of these agents will also deplete T cells [47]. The anti-CD20 monoclonal antibody rituximab is more specific to B cells but has the therapeutic limitation that the terminally differentiated antibody producing plasma cell does not express CD20 [50].

The development of potent anti-CD19 antibodies may offer an improved efficacy as CD19 is expressed both earlier in the B cell lineage compared to CD20, but has also been shown to deplete bone marrow residing long-lived plasma cells by up to 50% [51, 52].

Specific depletion of the plasma cells is an attractive target for specific immune depletion and the proteasome inhibitor Bortezomib is currently being assessed for its ability to both deplete HLA-specific antibody pre-transplant, but also to treat rejection episodes post-transplant [53-55]. When plasma cells manufacture IgG a proportion of the protein will be mis-folded, and under normal conditions this mis-folded protein is degraded within the proteasome and then recycled within the cell. Bortezomib prevents the degradation of mis-folded proteins which then build up within the cells and lead to apoptosis of the plasma cell. Recent studies have shown that Bortezomib can lead to modest reductions in circulating levels of HLA-specific antibodies in sensitized patients [56], but it appears unlikely that Bortezomib will be suitable for use as a desensitizing agent when given in isolation [57, 58]. Further studies have suggested that Bortezomib may have potential to treat AMR post-transplant [47].

1.4 The Major Histocompatibility Complex (MHC)

1.4.1 The Major Histocompatibility Complex (MHC): Structure

As has been discussed earlier, T cells only recognize antigens that have been degraded and presented by class I and II MHC molecules. The molecules of the major histocompatibility complex play a fundamental role in the immune system, primarily allowing the distinction to be made between self and non-self.

The MHC, or its equivalent, is a set of genes found in all species of vertebrates first discovered by Peter Gorer in the 1930s when he was studying antibodies to red blood cell antigens in mouse models [59]. Subsequently, George Snell coined the term histocompatibility (H) antigen to describe antigens capable of provoking graft rejection and that differences in the H-2 antigen first described by Gorer provoked the strongest graft rejection response [15]. The human MHC was subsequently described by Jean Dausset in the 1950s [17].

The human MHC is located on the short arm of chromosome six and comprises of two sets of genes encoding the human leukocyte antigen (HLA) system which lie either side of a genetic region (often referred to as the MHC class III region) which includes a range of genes encoding proteins involved in immune regulation (figure 1.8).

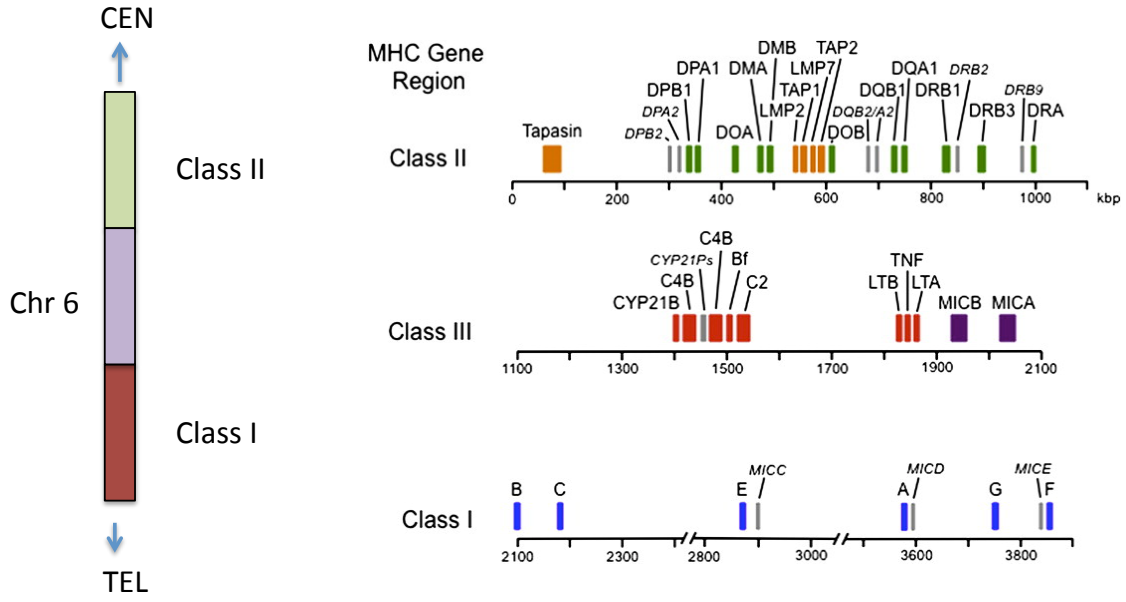


Figure 1.8: Map of the human major histocompatibility complex on chromosome 6. Class I HLA (HLA-A,B,C) loci and class II HLA (HLA-DR, DP, DQ) are encoded alongside a range of genes encoding proteins involved in immune responses (e.g complement proteins, proteins involved in antigen processing and presentation).

The MHC class I region contains the genes required for HLA class I molecules. The HLA class I molecule consists of a heavy polypeptide alpha (α) chain of approximately 44kDa which is noncovalently linked to a 12kDa polypeptide called β_2-microglobulin. The heavy chain is organized into three extracellular domains (α_1, α_2, and α_3), that are anchored to the cell surface by a hydrophobic anchor region. A short hydrophilic sequence carries the C-terminus end of the molecule into the cytoplasm (figure 1.9).

The HLA class II molecule is also a transmembrane glycoprotein, but differs structurally from the class I molecule as it lacks β_2-microglobulin and instead consists of two polypeptide chains (α and β) which are 34 and 29kDa respectively, and ordered

into membrane distal α_1 and β_1 domains above the membrane proximal α_2 and β_2 domains (figure 1.9).

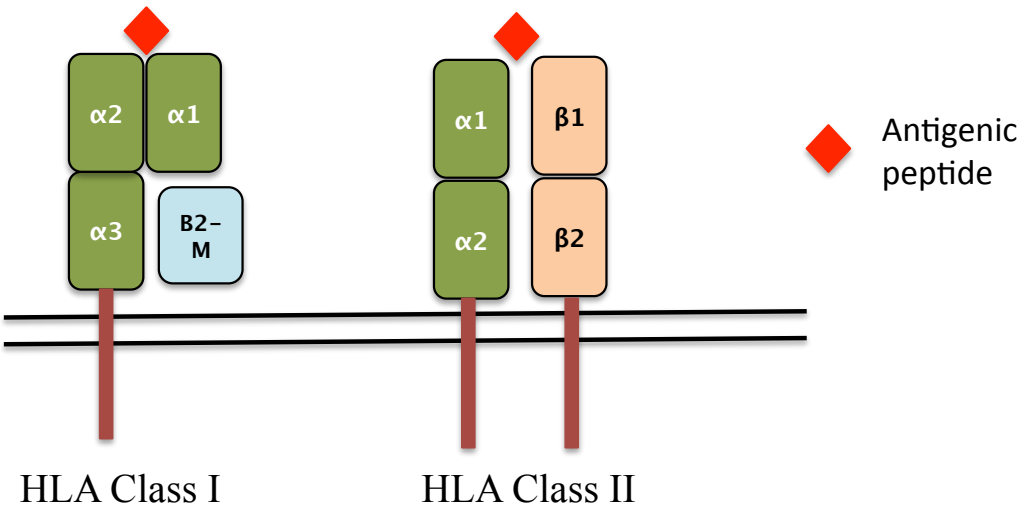


Figure 1.9: Basic structure of the class I and class II HLA molecules. The antigen binding portion consists of α_1 and α_2 domains for class I and α_1 and β_1 domains for class II in association with antigenic peptide.

There is significant structural homology between class I and class II HLA molecules. This is particularly apparent in the membrane distal portion of both class I and class II HLA molecules, the region where peptide presentation and T cell recognition occurs. In the class I molecule the α_1 and α_2 domains combine to form two alpha helices which lie parallel to each other and above a β-pleated sheet 'floor' to produce a very distinct 'groove' into which an antigenic peptide is situated and held in position due to the presence of conserved 'anchor' residues in the peptide sequence. The class II molecule forms the groove in a similar way by combining the

α_1 and β_1 domains. This structure in association with the antigenic peptide is often said to resemble 'sausages on a barbeque' (figure 1.10). The nature of the peptide binding groove differs slightly between class I and class II MHC. The peptide binding groove of the class I molecule is closed at either end and allows the binding of an antigenic peptide typically 8-10 amino acids in length. The class II binding groove is significantly more 'open-ended' allowing for a greater degree of peptide binding flexibility with 10-13 amino acid peptides typically accommodated.

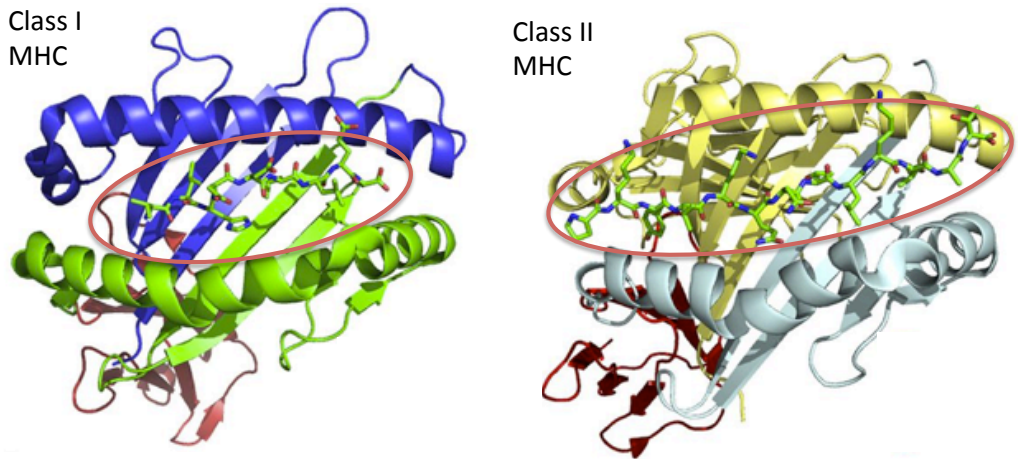


Figure 1.10: The peptide binding groove of the MHC class I and class II molecules. The antigenic peptide can be seen between the two parallel alpha helices producing a recognition complex seen by the T cell (circled). The class II peptide binding groove is open-ended and allows for a longer peptide to be accommodated.

1.4.2 The Major Histocompatibility Complex (MHC): Function

The MHC molecule has a range of functions, the main one being to present peptides to T-cells, but they can also act as inhibitory ligands for NK cells via interaction with a range of NK cell surface receptors, most notably killer cell immunoglobulin-like receptors (KIRs) and the NKG2A receptor [60, 61].

The MHC class I molecule primarily presents antigen that derives from intracellular pathogen such as viruses. As viruses can attack virtually any nucleated cell the immune system responds by incorporating MHC class I expression on virtually all nucleated cells also. The processed peptide is presented by the MHC class I molecule for recognition by the CD8 positive cytotoxic T-cell (Tc). The CD8 positive T-cell induces the death of the infected cell before the infected cell can multiply. Initially the naïve cytotoxic T-cells are activated when the T-cell receptor (TCR) recognizes and interacts with a peptide-MHC class I complex. The CD8 positive T-cell can induce apoptosis of the infected cell via the release of the granular components, perforins, granzymes, and granulysin. Upon recognition of target antigen, CD8 positive cytotoxic T-cells release perforins and granzymes onto the surface of the target cell. Perforin allows granzymes to enter the target cell and cause apoptosis [62].

Once the infected cells have been cleared the majority of the cytotoxic T-cells die and are cleared by phagocytosis. A small number remain to serve as memory T-cells. These memory T-cells can respond rapidly upon encountering the same antigen, differentiating into an effector cell population in a fraction of the time required following the primary response to antigen.

Class II MHC molecules are primarily expressed on professional antigen cells such as B lymphocytes, dendritic cells, and macrophages. Importantly, class II

expression can also be induced on capillary endothelial and epithelial cells by γ-interferon [63]. The function of the MHC class II molecule is to present antigen of extracellular origin to CD4 positive T-helper (Th) cells.

Antigen is internalized by endocytosis, processed, then presented by the MHC class II molecule. The TCR/CD3 complex of the T-helper cell binds to the peptide-MHC complex, this interaction is strengthened by the binding of the T-helper cell marker CD4 to a conserved region of the MHC class II molecule. This close interaction causes the intracellular kinases present on the TCR, CD3 and CD4 proteins to activate each other via phosphorylation. This in turn causes the activation of the major biochemical pathways within the T-helper cell, the so-called signal 1 of Th-cell activation. Following receipt of the initial activation signal the naïve T-helper cell then seeks a second confirmatory signal designed to ensure that T-cell activation is in response to foreign antigen and not due to auto-reactivity. This signal 2 involves an interaction between the CD28 molecule expressed on the T-helper cell and either the CD80 or CD86 protein expressed alongside the MHC class II complex on the antigen presenting cell [64].

Following the second signal the T-helper cell proliferates, mediated by a potent release of the growth factor interleukin-2 (IL-2). Activated T-helper cells also begin to express a fully functional form of the IL-2 receptor (IL-2R), so IL-2 acts in an autocrine manner to stimulate proliferation. The released IL-2 can act upon its cell of origin or an adjacent cells leading to a potent clonal expansion response. The activated T-helper cell can then further differentiate into Th-1 or Th-2 cells depending on its local cytokine environment. Th-1 cells are induced in response to interferon-gamma (IFN-γ), and Th-2 cells arise from interleukin-4 (IL-4) stimulation.

The Th-1 type response is characterized by the production of IFN-γ which in turn activates macrophages and allows them to mediate their potent bactericidal

properties. Th-1 cells also induce B cells to produce opsonizing antibodies, resulting in a powerful B-cell mediated immune response. Both Th-1 and Th-2 cells can provide the required co-stimulatory signals to B-cells in order that they can start producing antibodies by encouraging differentiation of B cells into antibody secreting plasma cells (figure 1.4). Signals derived from both Th-1 and Th-2 subsets can also insitigate class switching of activated B-cells to produce antibodies of different isotype (discussed in more detail in section 1.5).

In the context of transplantation differences in the MHC between graft donor and recipient can be detected by host T-cells. The two main routes by which foreign MHC is detected are by direct and indirect allorecognition (figure 1.11). Direct allorecognition occurs when host T-cells recognize an intact graft MHC molecule as foreign and mounts an immune response directly. The alternative route, indirect allorecognition, occurs when graft MHC is internalized and presented as antigen peptides in the context of recipient MHC on antigen presenting cells. Direct allorecognition is thought to be more important in acute rejection, particularly in the presence of MHC disparity between donor and recipient. Indirect recognition is thought to contribute to rejection by activating macrophages leading to fibrosis, although alloantibody is generated by the indirect pathway only [65].

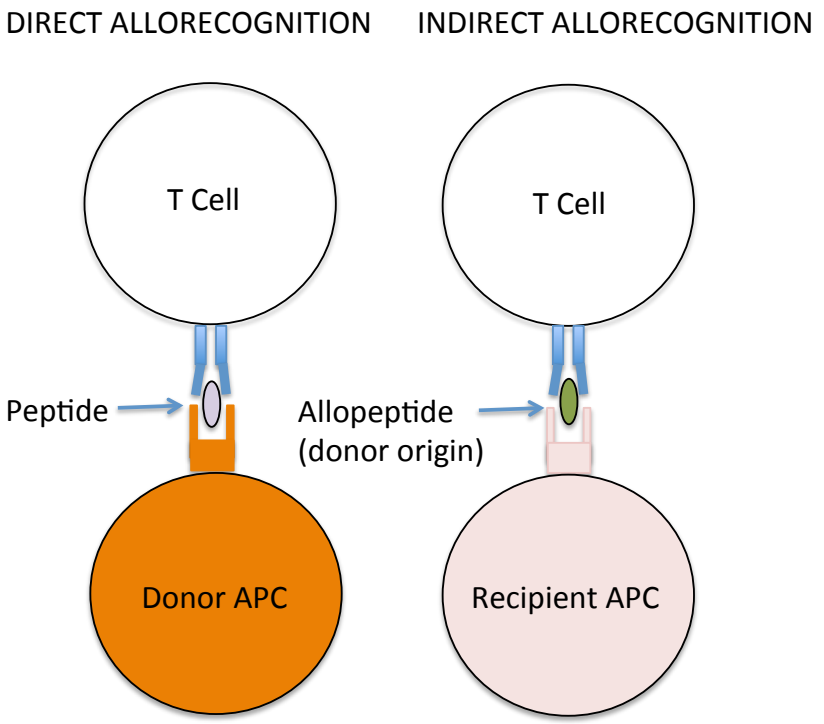


Figure 1.11: Direct and indirect allorecognition. Direct allorecognition: The whole donor MHC/peptide is recognized by the T cell. In contrast, for indirect allorecognition foreign MHC sequences are presented as peptides in the context of self-MHC.

1.4.3 The Major Histocompatibility Complex (MHC): Polymorphism

The MHC has evolved into a highly polymorphic system based upon multiple alleles at each locus. This evolution has most likely emerged as a consequence of pathogen-driven selection, with those alleles providing a 'survival advantage' for the individual being selected. For example, an allele which provides enhanced protection from an infectious organism would be selected. The class I and class II genes located on chromosome 6 are the most polymorphic in the entire human genome, with over 1200 allelic variants listed at some loci. All of these observations imply that the human MHC

has been under extreme evolutionary pressure with mutation rates of genes within this region far in excess of those at other genetic loci [66, 67].

The HLA-class I alpha chain molecules are broadly divided into the products of the HLA-A, B, and C genes and all are highly polymorphic. HLA-class II molecules exhibit virtually all of their polymorphism in the different beta-chain genes (HLA-DRβ, DQβ, and DPβ), with the class II alpha chain showing very limited polymorphism (table 1.3)[68].

Table 1.3: HLA Class I and Class II. Number of different protein variants for each locus identified to date. Source: HLA Nomenclature (http://hla.alleles.org), accessed 18th February 2013.

HLA Class I	
Locus	Number of allotypes
A	1571
B	2156
C	1252

HLA Class II	
Locus	Number of allotypes
DRB1	959
DRB3	46
DRB4	8
DRB5	17
DRA1	2
DQA1	31
DQB1	137
DPA1	18
DPB1	136

The amino acid differences that contribute to this polymorphism are largely restricted to the α1 and α2 domains of the class I molecule and the β1 domain of class II. Significantly most of these differences are located around the α-helices and β-pleated sheet structure that defines the antigen presenting region (figure 1.10). This is the region

of the molecule that directly interacts with the TCR, and further supports the observation that HLA-polymorphism occurs as a result of selection pressure and repeated host-pathogen interaction.

The genes of the MHC are inherited in a Mendelian co-dominant manner, with the whole haplotype inherited as a single trait, one from each parent and both are expressed. Infrequently, meiotic cross-over events and recombination events may occur to add to the complexity.

1.5 Antibodies

1.5.1 Antibodies: Structure and Function

As discussed previously, B cells can be activated following Th cell interaction with MHC/peptide complexes, and can differentiate into antibody secreting plasma cells (figure 1.4). The first function of an antibody is to recognize and to bind foreign material or antigen. This is achieved by recognizing molecular structures on the surface of foreign material termed epitopes which differ from those of the host. The human immune system is capable of raising a specific antibody response to billions of different molecular structures, and the mechanisms by which this is made possible are discussed later.

Antibodies, or immunoglobulins as they are also known, have a relatively simple structure consisting of two identical units involved in antigen binding,- the Fab fragments (fragment for antibody binding). These are the regions that confer the binding specificity on the antibody molecule, and as such contains regions of amino acid sequence variation. The presence of two identical Fab fragments per molecules also

serves to enhance the binding ability to antigen. The 'tail' region of the antibody molecule is termed the Fc (fragment crystallizable) region, and is the portion of the molecule associated with effector functions (figure 1.12).

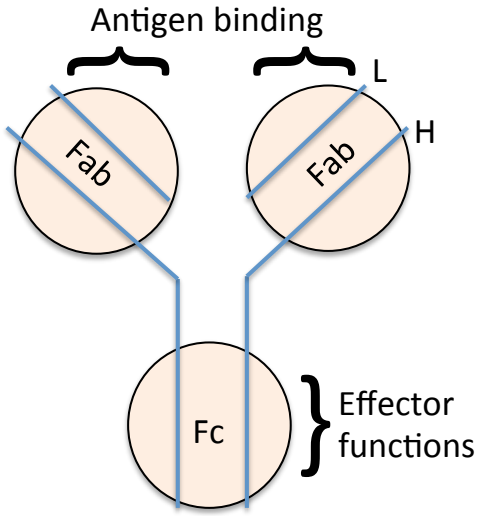


Figure 1.12: Simplified diagram of antibody structure. Two identical heavy chains (H) and two light chains (L) combine to make the structure shown.

The fine structure of the immunoglobulin monomer was elucidated by Rodney Porter and Gerald Edelman [69], and in the 1980s both were jointly awarded the Nobel prize for their contribution in this area. By selectively breaking down the disulphide bonds which hold together the immunoglobulin structure it could be clearly demonstrated that the molecule consisted of four chains. By the addition of papain the molecule was broken down into the Fc and individual Fab fragments. Treating with pepsin gave Fab_2 and a truncated Fc portion (figure 1.13).

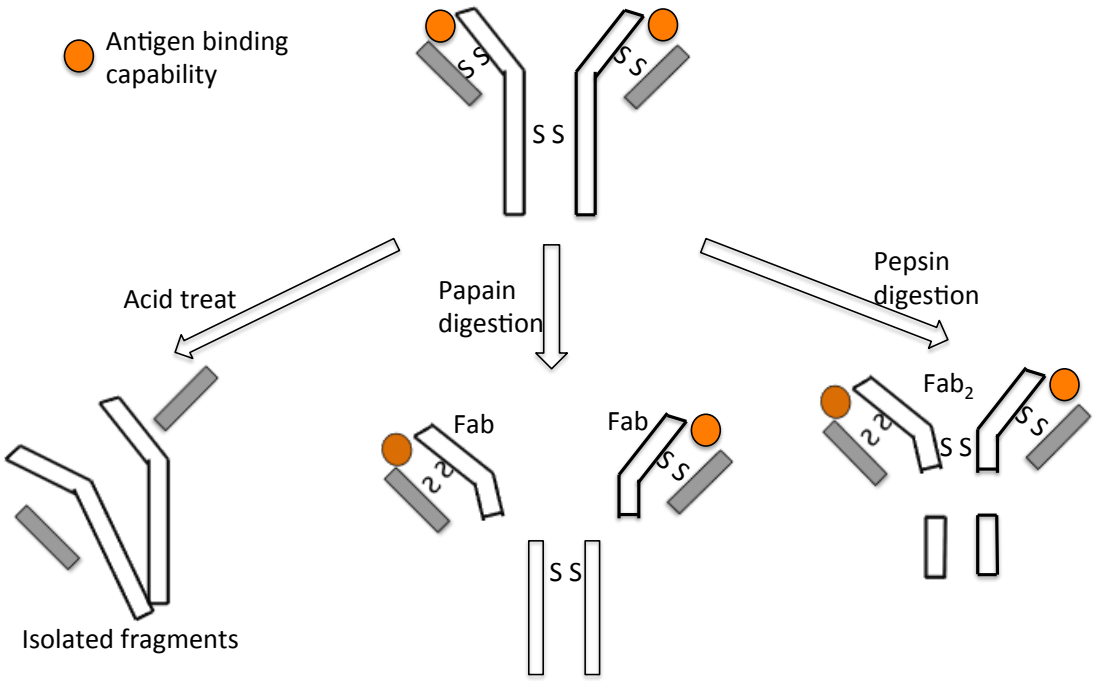


Figure 1.13: The structure of the immunoglobulin monomer elucidated by Porter and Edelman [69]. Proteolytic cleavage of the molecule by papain and pepsin; selectively cleaving with papain produces two separate Fab fragment capable of antigen binding but the molecule is no longer able to precipitate antigen. Pepsin digestion led to the production of a Fab_2 fragment which retained both binding sites and thus maintained the ability to precipitate antigen.

The process by which antibody binding diversity is produced is via a complex system of gene rearrangements involving the genes encoding the heavy and light chains of the immunoglobulin molecule (figure 1.12). The variable region of the human heavy (H) chain contains three gene segments termed V (variable), D (diversity), and J (joining), whereas the light (L) chain contains V and J only. There are multiple human heavy and light chains (table 1.4), and these can undergo recombination to produce many different combinations (figure 1.14). Even more diversity can then be achieved

by the different pairing of heavy and light gene, as any heavy chain can pair with any light chain.

Table 1.4: Approximate numbers of potential gene segments encoded human immunoglobulin heavy and light chains.

Heavy Chain (H)			Light Chain (L)	
V	D	J	V	J
50	27	6	35	5

To further increase the potential diversity, post-recombination modifications can be made to the heavy and light chain genes. Following recombination, nucleotides can be added or subtracted at the 'joins' between the gene segments, for example P-nucleotides can be added when uneven cleavage occurs at the site of recombination. Similarly addition of extra nucleotides, termed N-nucleotides by the enzyme terminal deoxynucleotidyl transferase (TdT) also enhances this junctional diversity.

Following the production of a functional antibody molecule further diversity can be achieved via somatic mutation of the V gene of the heavy and light chains. Point mutations in the V regions of rapidly proliferating B-cells can occur in the germinal centres of lymphoid follicles, and as such is antigen-driven. This mutation can often result in a mutated immunoglobulin with an even higher affinity for its cognate antigen. These antibodies are then able to better compete for the limited amounts of specific antigen and become the dominant antibody, thus raising the overall affinity of the immune response in a process known as affinity maturation.

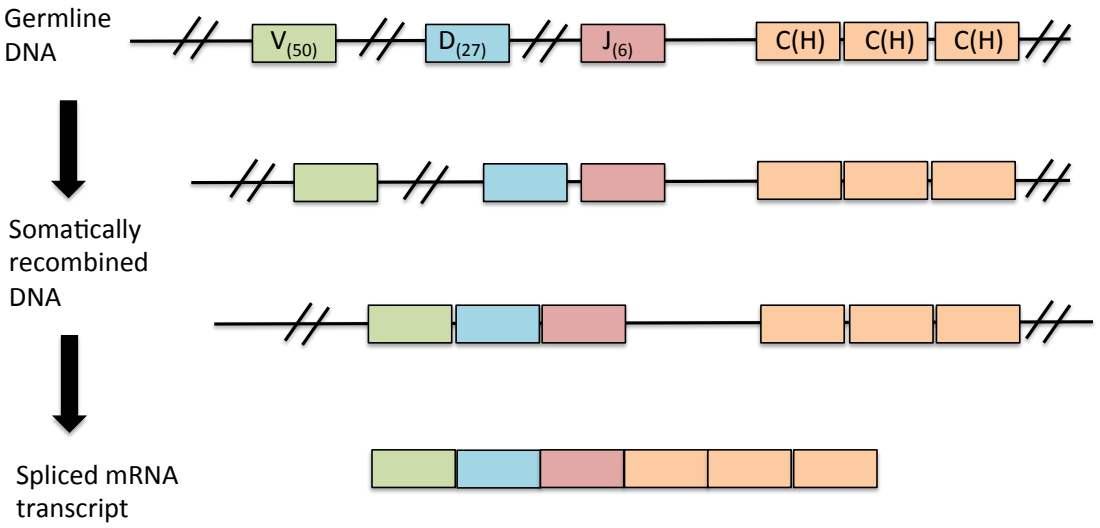


Figure 1.14: Overview of heavy chain (H) recombination. Diversity (D) and Joining (J) gene segments are joined first, then a Variable (V) gene segment is added to produce the fully recombined heavy chain gene.

There are five classes of immunoglobulin termed Immunoglobulin G, (IgG), IgM, IgA, IgE, and IgD. All classes share the same four-chain antibody structure as outlined in figure 1.12. However, key differences between the classes are observed in the heavy chain with the most pronounced situated in the Fc portion (table 1.5). The Fc portion is crucial to the activation of effector mechanisms upon binding to antigen. The various effector mechanisms favoured by each subclass is listed in table 1.5.

Table 1.5 Summary table of the human immunoglobulin classes.

Ig Class	Subclasses	Molecular Form	Location (Concentration)	Complement Activation	Properties
IgG	IgG1	Monomer	Serum (~9mg/ml)	[++]	Major immunoglobulin of acquired
	IgG2	Dimer	Serum (3mg/ml)	[+-]	immunity
	IgG3	Monomer	Serum (~2mg/ml)	[+++]	
	IgG4	Monomer	Serum (~1mg/ml)	[-]	Can cross the placenta
IgA	IgA1	Monomer	Serum (3mg/ml)	[+]	Found in gastrointestinal,
	IgA2	Dimer	Secretions:	[+]	respiratory, and genitourinary
		Secretory	milk, colustrum, tears		tract mucosa
IgM		Pentamer	Serum (~1.5mg/ml)	[++++]	Secreted early in acquired immune response
IgE		Monomer	Serum (~0.05ug/ml)	[-]	Involved in allergry and anti-paristic response
IgD		Monomer	Serum (~30ug/ml)	[-]	Membrane bound immunoglobulin

The different immunoglobulin classes have distinct roles to play in the immune system and this is reflected by the differences in their respective structures. If we consider them individually in the order of their concentration in human serum this can be explained.

Immunoglobulin G (IgG) is the most prominent antibody class in serum and other mucosal tissues. It is able to coat target cells and promote a stable interaction with phagocytic cells in a process known as opsonisation. IgG can also eliminate antigen through activation of the complement system via the classical pathway (explained in section 1.6), an effector mechanism which is dependent on the particular subclass of IgG involved. As shown in table 1.5 IgG1 and IgG3 are the most potent activators of the complement cascade, with IgG2 having little or no complement activating capacity, and IgG4 none at all. IgG can also interact with various Fc portion specific cell surface molecules (Fc receptors). Fc receptors typically consist of a ligand binding α-chain, usually complexed with a transmembrane region that is a dimer of the common γ-chain (FcRγ). Binding of Ig to Fc receptors activates various cell signaling functions, for

example binding of IgG to FcγRI expressed on monocytes, macrophages and dendritic cells results in enhanced phagocytosis, up-regulation of antigen presentation, and in the mediation of extracellular killing of target cells referred to as antibody-dependant cellular cytotoxicity (ADCC).

IgM is pentameric in structure and is highly effective at complement activation as a consequence of this (see section 1.6). IgM is also the major class of antibody associated with a primary immune response.

IgA is found in three distinct soluble forms (table 1.5), with monomeric and dimeric (linked by a polypeptide known as the J chain) found in the serum. These antibodies complex pathogens to effector cells via interaction with IgA specific Fc receptors. Secretory IgA is a complex of dimeric J chain-linked IgA and a secretory protein and is a key defence mechanism against microbial attack of the human mucosal surfaces. IgA has two subclasses IgA1 and IgA2, the shorter IgA2 molecule is more resistant to attack from proteases released by bacterial pathogens.

IgE is monomeric and found at low concentrations in the serum, probably due to the fact that most IgE is bound to IgE specific Fc receptors expressed by mast cells. IgE is the major class of antibody associated with an acute inflammatory response, where antigen binding to Fc-bound IgE results in cross-linking and a potent degranulation of mast cells leading to a powerful inflammatory response. This often leads to unwanted hypersensitivity in response to certain antigens, resulting in a state of allergy. Finally, IgD is an antibody class found primarily on the B-cell surface where it is thought to be involved in some way in the control of lymphocyte activation.

The production of antibodies of the various different classes occurs at different rates in a distinct order governed by gene order and under strict T-lymphocyte control. This is demonstrated in mouse models where T-cell deficient mice show greatly reduced degrees of immunoglobulin class switching [70]. The early antibody response

is typified by a powerful IgM response which usually falls off rapidly to be replaced by a more gradual and sustained IgG response, which results in much higher IgG titres. Therefore in the process of class switching the variable (antigen-binding) region of the molecule remains unchanged but the Fc region is altered, thus the specificity remains but the potential effector function of the antibody is altered. Figure 1.15 shows the order of the human immunoglobulin heavy chain genes on chromosome 14.

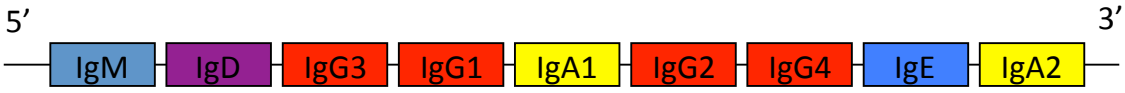


Figure 1.15: The order of immunoglobulin heavy chain genes on chromosome 14. This is the usual order of antigen specific antibody maturation, leading to an adaptation of the antibody mediated immune response [71].

Activated B cells migrate from the bone marrow to peripheral lymphoid organs such as the spleen, or gut-associated lymphoid tissues (GALT), and undergo antigen driven clonal expansion (figure 1.4). At this point B-cells may undergo class switching via a process termed class switch recombination (CSR) in response to signals generated by extracellular cytokines [72]. CSR may occur via a T-cell dependent mechanism involving the interaction between the CD40 cell membrane protein expressed on B-cell with the CD40 ligand (CD40L) on T-cells, or by CD40 independent mechanisms involving direct super-antigenic stimulation [73]. Superantigens are substances capable

of binding to the Vβ domain of the TCR and residues in the alpha chain of the class II MHC molecule simultaneously resulting in T-cell activation.

Class switching uses distinct regions of the gene sequence to achieve recombination. During CSR portions of the heavy chain gene sequence are deleted from the chromosome at specialized 'switch regions' (S regions) by the actions of a number of enzymes such as Activation Induced Deaminase (AID), Uracil DNA glycosylase [74]. Regions surrounding the deleted sequence are rejoined such that a functional gene is produced but the heavy chain isotype has been substituted via a process known as non-homologous end-joining (figure 1.16).

The immunoglobulin class of a particular antibody has a profound effect on the functional capability of the molecule. The ability of an antibody to mediate targeted cell destruction via the activation of the complement cascade is entirely dependent upon its isotype.

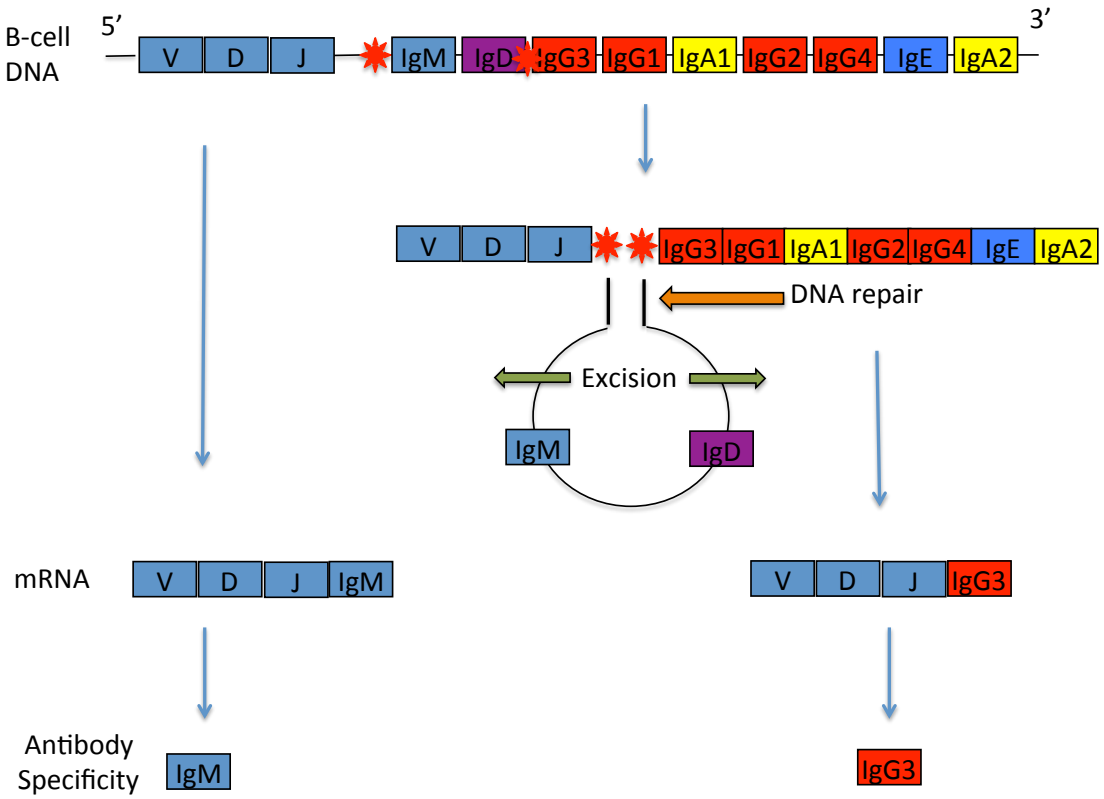


Figure 1.16: Immunoglobulin heavy chain class-switching. A T-cell regulated process of DNA excision and repair leads to the formation of a new antibody with altered heavy chain usage but maintaining the original binding specificity. Breaks in the DNA are initiated at specific 'switch' points (red asterix). Binding specificity is denoted by the blue V,D,J gene sections [73].

1.6 The Complement System

The complement system is comprised of a group of around 30 plasma proteins that can become activated and then interact in a powerful cascade that culminates in the production of a potent immunological complex capable of rapid and widespread target

cell lysis. However, in addition, as part of the complement cascade various components can fulfill a range of immune effector functions. There are three routes by which the complement system can be initiated, the classical pathway, the alternative pathway, and the mannose binding lectin (MBL) pathway. Complement factors are given the letter C followed by a number, with the factors named in their order of discovery rather than sequence of reaction. Thus the sequence of reaction is C1, C4, C2, C3, C5, C6, C7, C8 and C9. Cleaved products are designated a lower case letter following the number with the letter "a" for the smaller product and letter "b" for the larger product (the exception to this is factor C2 where the larger fragment is factor C2a and the smaller fragment C2b). The larger fragments have specific enzymatic activity resulting in cleavage of subsequent complement proteins. Alternative pathway factors are given letters instead of numbers, for example factor B and factor D. In the MBL pathway, the initial enzymes to be activated are called MASP-1 and MASP-2 (mannose-binding-lectin associated serine proteases). An overview of the pathways of complement activation are shown in figure 1.17. As figure 1.17 shows, although the pathways are each inititated differently they do converge into a common final pathway culminating in the formation of the C5-C9 membrane attack complex (MAC). The key to each pathway is to reach the point at which the C3 cleaving molecular complex is formed.

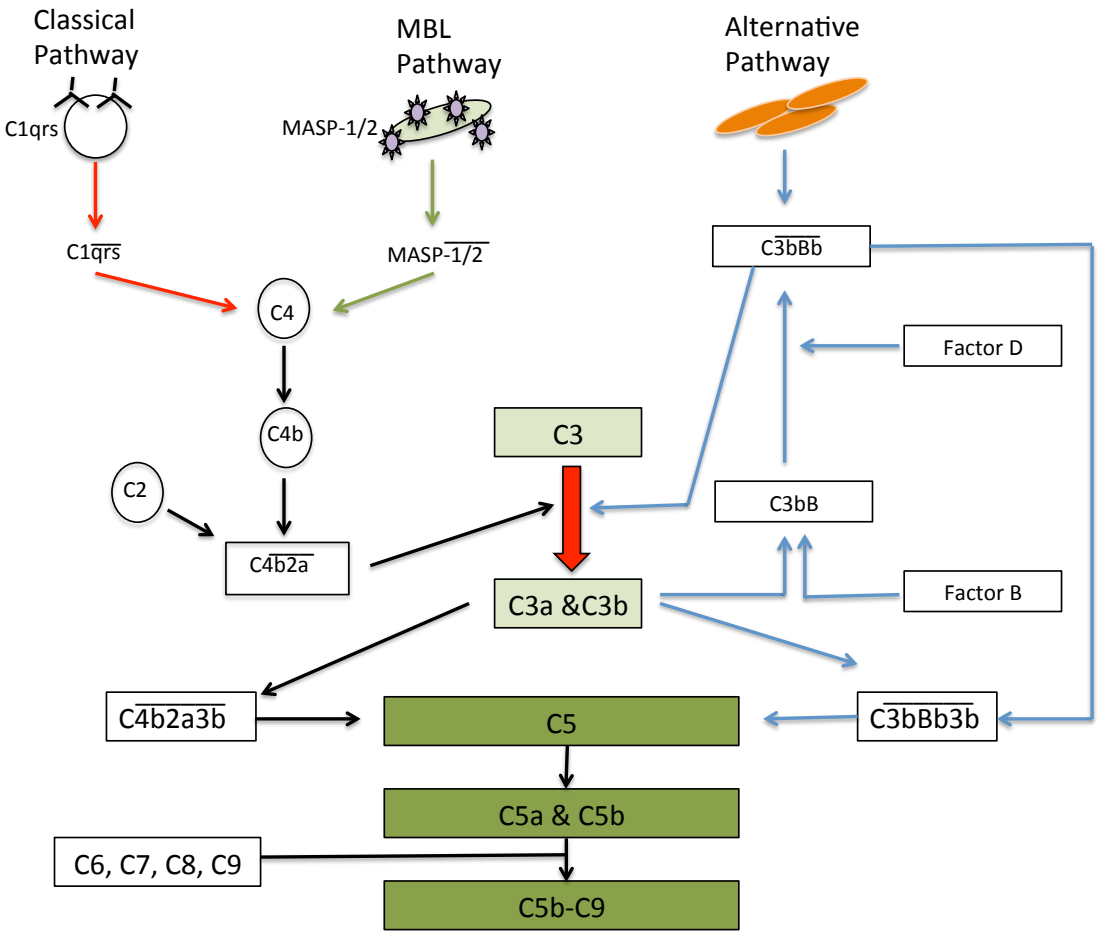


Figure 1.17: Simplified overview of the three pathways of complement activation. All three pathways converge onto the final common pathway. Classical and MBL pathways cleave C4 and subsequently C3. Protein complexes responsible for protein cleavage are marked with an overline.

The MBL pathway is initiated when MBL associates with two serine proteases called MASP-1 and MASP-2. The resulting complex is able to bind extracellular sugar moieties on the surface of pathogens, which in turn activates MASP-2 to cleave the C4 and C2 complement proteins to generate C4b2a [75]. C4b2a is the key C3 convertase molecule in the MBL pathway (figure 1.17).

The classical pathway is initiated by an antibody-antigen interaction and involves the C1q hexameric complex C1qrs (figure 1.17). When the C1qrs complex is cross-linked with at least two immunoglobulin Fc portions of pathogen bound antibody it induces a conformational change in the C1qrs complex resulting in autolytic cleavage of C1r and C1s. Cleaved C1s results in the formation of an active serine protease which in turn cleaves C4 generating C4b, which binds covalently to proteins or carbohydrate on the surface of the pathogen. To prevent an auto-reactive response unbound C4b is quickly inactivated. Pathogen bound C4b then binds to the C2a fragment of the C2 molecule, which is first cleaved by activated C1s, to form the C4b2a complex, or C3 convertase.

The alternative pathway was discovered after the classical pathway and is an alternative means of complement activation. The activation of the alternative pathway does not depend on the presence of proteins such as immunoglobulins or MBL, and is more likely to be initiated following interaction with immune complexes. As shown in figure 1.17 the classical and MBL pathways are activators of the alternative pathway as they are able to generate C3b. The alternative pathway begins with a low rate of spontaneous hydrolysis of plasma C3 to form C3(H2O). C3(H2O) has an altered conformation which binds to factor B. Which in turn is then cleaved by factor D to release Ba and form a fluid-phase C3(H2O)Bb complex. The C3(H2O)Bb complex is a C3 convertase and cleaves C3 to C3a and C3b most of which is rapidly inactivated. Some C3b molecules attach to pathogen or host cell surfaces and bind factor B. Factor B is cleaved by factor D to form Ba and C3bBb. The bound C3bBb molecule is called the alternative pathway C3 convertase and functions in much the same way as C4b2a in the classical and MBL pathways.

Each pathway then follows a common final pathway, which is initiated when C3 convertase is complexed with the C3b fragment to form C5 convertase (figure 1.17).

This step is also a key amplification stage with C3 being by far the most abundant complement protein. Cleavage by C5 convertase results in C5b which binds C6 to form C5b6 which in turn binds C7, and the resulting complex is then able to insert itself into the cell wall following a conformational change which exposes a hydrophobic residue on the C7 protein. C8 then binds to the C5b portion of the C5b67 complex and recruits and polymerises up to 16 molecules of C9 to form the MAC. The MAC has a hydrophobic external surface which allow it to insert itself into the lipid bilayer of the cell membrane. A hydrophilic inner channel with a diameter of around 100 Å then allows the free passage of water and other solutes resulting in rapid and potent cellular destruction [76].

In addition to the formation of MAC, the cleaved products of all pathways of complement activation, in particular following C3 and C5 cleavage, fulfill a variety of immunological functions augmenting the potency of the complement system. Some of these are summarized in table 1.6.

Table 1.6: Immunological roles of various complement cascade proteins.

Complement Protein	Immunological Effect	Main Target
C3a	Mast cell degranulation	Mast cell
C3b	Opsonisation Immune complex clearance	Pathogen surface Red blood cell
C3d	Mast cell degranulation B-cell activation	Mast cell B-cell
C4a	Mast cell degranulation	Mast cell
C4b	Opsonisation	Pathogen surface
C5a	Mast cell degranulation	Mast cell
C5-C9	Cell lysis	Infected cell

1.7 HLA Specific Antibodies

1.7.1 HLA Specific Antibodies: Specificity

HLA-specific antibodies recognise epitopes, and the definition of an epitope can be given as: 'A localised region on the surface of an antigen that is capable of eliciting an immune response and of combining with a specific antibody to counter that response'. So HLA-specific antibodies recognise a region of the HLA molecule that is primarily non-self, but more importantly may be shared amongst many HLA. Recent studies have also shown that hydrophobicity and electrostatic charge of the epitope defining residues play a key role in antibody recognition and binding [77, 78].

Much recent work has been performed in this area to identify potential immunogenic sites on the HLA molecule against which a HLA-specific antibody can be raised. Worldwide registries have been set up that aim to document the currently identified repertoire of HLA epitopes, and to establish a common nomenclature for identifying these antibodies. Presently, epitope designations are based upon three-dimensional molecular modeling of the HLA protein and the amino acid residues found in polymorphic positions. Typically these epitope reactivates are determined using single antigen bead panels, which themselves have technical problems as will be discussed in section 1.10. As of 2012 there were 69 verified HLA-class I specific epitopes, 53 HLA-DR, 17 HLA-DQ, and 8 HLA-DP, although as will be discussed further in chapter 3 there are likely to be many more epitopes identified and verified in the future [79, 80]. Computer algorithms have been designed to allow the HLA-technician to interpret HLA antibody assay data in terms of epitope structure, the HLAMatchmaker program is a free to use algorithm (www.hlamatchmaker.net) that

will provide a likely epitope level interpretation for any HLA-specific binding profile [81-87].

To add further complexity to the epitope specific nature of antibody recognition it has also been demonstrated that some HLA-specific antibodies have contact residues on the HLA-bound peptide (see figure 1.9) [88]. This may cause problems in identifying these antibody specificities in the single antigen bead assays, where bound peptide repertoires are likely to differ significantly from those found in the individuals and binding may be prevented.

It is important to consider the epitope recognizing surface of the antibody molecule, which typically has three heavy chain regions and three light chain regions that form the primary 'contact surface' with antigen. These regions, termed complementarity determining regions (CDRs) bind to the 'functional epitopes' on the antigen which typically comprise 15-30 amino acid residues covering an area in the region of 700-1000Å [89].

Using the three-dimensional HLA modeling software program, Cn3d (available online: www.ncbi.nlm.nih.gov/Structure/**CN3D**/**cn3d**.shtml), it is possible to examine the structure of the molecule and how the epitope is situated and presented to antibody. Figure 1.18 shows the position of the common HLA-A2 specific epitopes designated 62G and 142T [80].

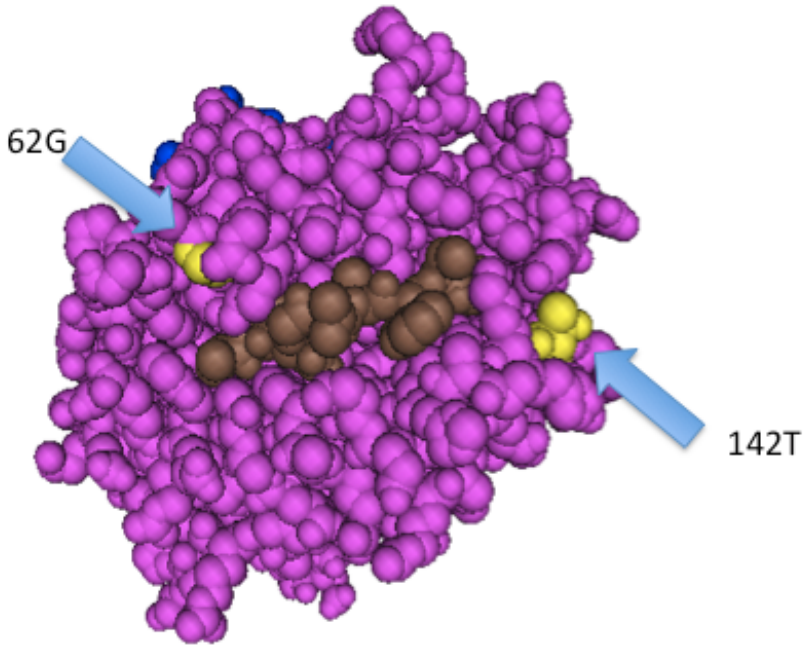


Figure 1.18: Three-dimensional structure of the HLA-A2 molecule (top-view). The 62G and 142T epitopes are labeled and sit either side of the bound peptide (brown).

1.7.2 HLA Specific Antibodies: Role in antibody-mediated rejection

HLA-specific antibody can be stimulated in response to graft mismatched HLA antigen, paternal mismatched antigens through pregnancy, and via HLA antigens expressed on donor cells following blood transfusion.

Hyperacute antibody-mediated rejection (AMR) due to HLA-specific antibodies was first recognized in the 1960s [24]. The introduction of the cytotoxic crossmatch test that same decade drastically reduced the incidence of hyperacute rejection [25]. Over recent years, in the context of antibody incompatible transplantation, AMR has again become a major challenge to successful renal transplantation[90-92].

AMR is a consequence of the interaction between donor HLA-specific antibodies (DSA) and the HLA expressing cells of the graft vascular endothelium. The binding of DSA on the graft endothelium can lead to localized classical pathway complement activation and subsequent cell death leading to ischaemic injury to parts of or the entire donor kidney [93]. Consequently, the endothelium loses integrity and free passage of coagulation factors ensues leading to vascular thrombosis [94].

AMR can be either acute or chronic, and is often associated with detectable levels of circulating DSA [95]. To diagnose acute AMR in a renal transplant biopsy there are a number of key histological features which are mandatory. The main feature of unequivocal AMR diagnosis is capillaritis, often in conjunction with glomerulitis and presence of circulating antibodies specific for donor HLA [96]. The identification of C4d has also emerged as a marker for AMR in the clinical transplant setting, which suggests that antibodies may not be the sole mediator of acute or chronic rejection [97]. Current treatment protocols for acute or chronic AMR are not fully effective, with various agents used in a combination of dosages. For example, one study showed that post-transplant intravenous immunoglobulin (IVIg) in highly sensitized patients lead to improved one-year survival outcomes and lower levels of HLA-specific antibodies [98]. Other studies demonstrated the benefit of multiple treatments of plasmapheresis to reduce DSA levels leading to more effective and reproducible reduction of humoral rejection rates when compared to IVIg [99]. The introduction of the proteasome inhibitor bortezomib, the therapeutic agent used to selectively target plasma cells discussed in section 1.3.6, is the first agent to effectively shut down antibody production in vivo [100]. However, studies have indicated that as antiviral antibody levels stay stable following bortezomib treatment it may not be as effective as first hoped on the long-lived bone marrow niche plasma cells [57].

Some centres have reported cases where the use of the monoclonal antibody specific for the C5 protein of the complement cascade eculizumab has been indicated to treat rejection. Binding of eculizumab to the C5 protein inhibits formation of the membrane attack complex (MAC) as outlined in section 1.6 and prevents complement mediated damage to graft tissue. One case study detailed the use of eculizumab in combination with IVIg and plasmapheresis to salvage a kidney graft undergoing severe AMR [101]. Locally we have published data describing the use of eculizumab to treat AMR following three separate HLA incompatible transplants [102]. From this data we concluded that although the use of eculizumab was not completely successful in abrogating the rejection, particularly in the face of extremely high DSA levels, a positive effect of eculizumab could be seen in all three cases. Therefore, eculizumab, like bortezomib discussed in more detail in section 1.3.6, constitutes a useful addition to the therapeutic repertoire used to treat AMR. Further randomized clinical trials are required to fully assess the efficacy of eculizumab.

1.8 Transplanting the sensitised patient

Aside from the chronic lack of available donors there are a number of other factors that can preclude renal transplantation. These can include patient age, presence of other co-morbid factors (heart failure, diabetes, obesity), or even religious or cultural beliefs, which can prevent the receipt of a donor organ. However, by far the most common preventative factor to renal transplantation is an immunological one- namely the presence of antibodies in the patients' blood. The most frequently encountered antibodies are those directed against blood group ABO and non-self HLA. It is estimated that around 250 transplants per year are prevented in the UK due to the

presence of an antibody incompatibility. Antibodies directed against a transplanted organ can cause rejection, this rejection may occur in different phases depending on the titre (strength) and specificity of the antibody:

1. Hyperacute rejection: graft destruction occurring within minutes of graft reperfusion. Histologically, the markers associated with HAR are neutrophil and platelet margination in the glomerular and peritubular capillaries leading to acute tubular injury.
2. Accelerated acute rejection: Distinct from hyperacute rejection and usually occurs within 2-3 days as a consequence of a specific memory T-cell response.
3. Acute rejection: distinct from hyperacute rejction as graft rejection usually occurs within the first two weeks following transplantation. Histologically the central feature of acute rejection is injury to the endothelium within the microvasculature.
4. Chronic rejection: progressive loss of graft function which may occur months or years after transplantation. The histologic features of chronic rejection are namely glomerular basement membrane duplication (chronic transplant glomerulopathy) and/or arterial intimal fibrosis.

The fate of the sensitised patient can be placed into one of the following categories:

1. Continue to wait for a deceased donor transplant. This option may be suitable for patients who have an ABO antibody barrier with their living donor but are negative for anti-HLA antibodies. For patients with an ABO barrier and broadly reactive HLA-specific antibodies this may not be a suitable choice as the chance of receiving an offer of a cadaveric kidney would be very low.

2. Look for an alternative live donor. Other potential live donors could be approached, some of whom may turn out to be suitable for standard live donor transplantation.

3. Enter the paired exchange transplant program. When histocompatibility testing has identified a barrier to transplant with a prospective live donor it is then common practice to advise these patients to register for a possible exchange transplantation. In exchange transplantation, recipients who have antibody-incompatible donors are entered into a donor-recipient matching pool. These incompatible pairs are then matched to other incompatible pairs such that new antibody-compatible transplant pairs can be created. This approach was first performed at the Johns Hopkins University in 2005 [92] and has also been successfully performed in the Netherlands [103], with the first exchange transplantation performed in the UK in 2007.

If none of the above options are suitable, then the option of proceeding to antibody incompatible transplantation incorporating antibody removal therapy (desensitisation) can be explored.

1.9 Antibody Incompatible Transplantation

Antibody incompatible transplantation (AIT) can be successful following the reduction of potentially graft destructive antibodies in the patient serum prior to transplant. There are a number of approaches that have been used historically to reduce the circulating levels of anti-HLA antibodies**.** Some transplant units use combinations of IVIg or the B cell specific monoclonal antibody rituximab, however these treatments are much less effective when patients have particularly high starting levels of antibody [104, 105]. It is therefore often preferable to use antibody removal therapy. These therapies can be broadly divided into the following main approaches:

Plasma Exchange.

Patient blood is passed through a plasma filter or is simply centrifuged. The plasma component, which contains the antibodies, is removed and discarded. However, along with the antibodies the plasma component also contains albumin and other clotting factors and these have to be replaced with fresh donor plasma. This treatment has been used clinically in other scenarios, notably to remove antibody of vasculitis triggering antibody in conditions such as renal vasculitis [106]. Depending on the size of the patient, only 3-4 litres of plasma can be treated per session[107]. In addition the efficacy of plasma exchange is limited as a significant proportion of antibodies are found in the extravascular space. Furthermore antibody can be re-synthesised between plasma exchange treatments further decreasing treatment efficacy.

Double-filtration plasmapheresis (DFPP).

Plasma is separated as for plasma exchange but is then passed through a second filter which operates via size exclusion. This filter traps larger molecules and consequently smaller plasma proteins which need to be replaced in standard plasma exchange such as albumin and lower molecular weight clotting factors are allowed to pass back into the patient. Although some clotting factors are still depleted potentially increasing the risk of bleeding during subsequent surgery. This approach is much better tolerated by the patient and typically DFPP treatment volumes can be 6-8 litres per session, usually only limited by fluid shift out of the vascular compartment [108]. Care must be taken clinically to reduce the risk of infection following reduction of antibody mediated immunity, and also DFPP can be unsuitable for patients with compromised cardiac function[107].

Protein A immunoadsorption.

Once again plasma is removed as for plasma exchange, but is then passed through a column containing immobilised Protein A. Protein A is a 56kDa protein derived from the cell wall of the bacterium *Staphylococcus Aureus* which is able to bind immunoglobulins (Ig) from many different mammalian species [109]. Protein A binds effectively to human IgG (though not to IgG3 as will be discussed further later), so is a highly effective method for removing antibody prior to transplantation. Following passage through a protein A column only the antibody molecules are removed leaving all other plasma proteins unaffected which makes this a very well tolerated treatment modality, with treatment volumes of up to 40 litres in a single session possible [110]. Figure 1.19 shows the increased effectiveness of protein A immunoadsorption

compared to standard plasma exchange or DFPP. The major disadvantage of protein A immunoadsorption is the high cost associated with protein A column. In addition protein A does not bind to the potent complement fixing IgG3 isotype; the immunological significance of this molecule will be discussed in chapters 6 and 7.

Selective adsorption of ABO antibodies.

Plasma is passed through a commercially produced column containing immobilised blood group antigen, either blood group A or B depending on the blood group of the potential donor. Therefore the only component removed is the ABO blood group specific antibody that is bound to the column, everything else in the patient plasma passes back into the patient. As with protein A columns, this makes this procedure very well tolerated with up to 10 litres of plasma treated in a single session reported [111]. Despite the high cost associated with this treatment, these columns are a very attractive choice for clinicians to use in their ABO-incompatible transplant programs as the columns are highly specific and have a large capacity to deplete even high titre antibody.

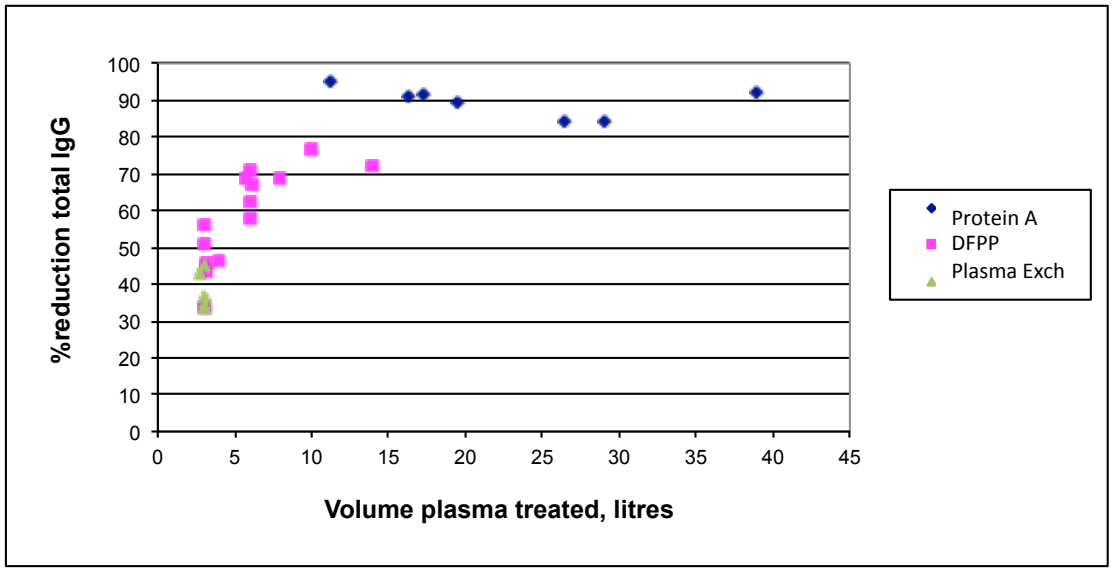


Figure 1.19: Single treatment effectiveness of three main approaches to reduce total IgG concentration in plasma. Protein A is by far the most effective method of antibody reduction, and combines removal efficacy with longer treatment duration.

The same approaches can also be used to remove antibody post-transplantation, although the clinical value of post-transplantation antibody removal is questionable and will be discussed further in section 1.10.

1.10 Laboratory techniques for defining risk in AIT

The ability to understand the degree of immunological incompatibility between donor and recipient is crucial in defining the level of risk prior to AIT. This can be determined in the laboratory by measuring the strength of anti-donor reactive antibodies using both highly sophisticated solid phase assays and traditional serological assays. The ability to measure the blood levels of donor-specific antibodies before and after the transplant is therefore critical.

The three commonly used methods for measuring HLA antibody levels are the complement dependant cytotoxic crossmatch (CDC), the flow cytometry crossmatch (FC), and HLA microbead analysis. The fundamental principle of each assay will be described here followed by a review of the potential clinical significance of each technique. For a conventional antibody compatible transplant donor/recipient compatibility is characterized by negative CDC and FC crossmatches and no detectable donor-specific antibodies (DSA) by microbead analysis. However, in the case of antibody incompatible transplantation these techniques can be used in combination to determine the risk to graft survival, when the transplant is performed in the presence of circulating DSA [91].

The Complement Dependent Cytotoxic Crossmatch (CDC)

The CDC crossmatch was first described by Terasaki and colleagues in the 1960s and was shown to be an accurate predictor of hyperacute rejection prior to transplantation [25]. Donor lymphocytes are typically separated into T and B cell compartments, and patient serum is mixed with the lymphocytes. A source of complement (typically rabbit serum) is added. If donor-specific antibody is present it will bind to donor cells and the complement cascade will be activated via the classical pathway and lysis of the donor lymphocytes will result. This can be assessed microscopically by comparing the percentage of dead to live cells, higher percentages of dead cells mean more lysis, which in turn means a higher titre of donor specific antibody. Highly cytotoxic patient sera can be tested at a range of dilutions to better determine the titre. The sensitivity of the CDC assay can be increased by the addition of anti-human globulin (AHG) which binds to the lambda light chains of the donor specific antibody resulting in an increased density of available Fc receptors which are

able to associate with complement component C1q and initiate the classical pathway [112].

The CDC crossmatch can give false positive results, either as a result of technical error or more likely the presence of autoantibodies in the recipient serum. To determine the presence of autoantibodies a separate crossmatch should be performed against the recipients own lymphocytes. In addition, autoantibodies tend to be IgM rather than IgG so the crossmatch should be routinely performed with test wells treated with dithiothreitol (DTT) a reducing agent to which IgM is particularly sensitive. Also, it is often the case that a CDC crossmatch can be negative in the presence of donor-specific antibody of low titre which is too low to reach the threshold required for complement activation. Additionally if the donor specific antibody is comprised of poor complement fixing ability isotypes (IgG2 or IgG4) then a negative result can be observed.

The Flow Cytometry Crossmatch (FC)

The introduction of the FC crossmatch in the 1980s successfully addresses the issue of complement fixing isotype composition and lower sensitivity encountered with CDC [113]. In this technique, patient sera is once again mixed with donor lymphocytes. Fluorescently labeled anti-human IgG monoclonal antibody is added to the mixture and will bind to any antibody bound specifically to donor cells. T cells and B cells can be distinguished by the addition of fluorescently labeled CD19/20 (B cells) or CD3 (T cells). Antibody negative blood group AB serum is used routinely as a negative control and positivity is determined by shift in median channel fluorescence away from the negative control. This technique has added sensitivity compared to CDC

and also directly detects all bound donor-specific IgG and is not reliant upon a secondary effector mechanism such as complement activation.

Microbead Analysis

This is a recently developed tool, which has made it much more practical to measure DSA on a daily basis. Unlike CDC and FC crossmatching, microbead analysis does not rely upon a constant supply of fresh donor lymphocytes to allow for daily monitoring. Instead, target HLA proteins are purified and attached to polystyrene beads [114, 115], the beads themselves are individually labeled with specific ratios of fluorescent marker dyes. The beads are incubated with patient sera and any HLA-specific antibody present will bind to the HLA protein coupled to the microbead. A fluorescently labeled anti-human IgG antibody is then added and then both the bead sets and the bound antibody are classified by two colour lazer analysis on the Luminex™ platform (Figure 1.20).

The beads shown in figure 1.20 are commonly referred to as phenotype beads as they carry entire HLA haplotype profiles. More recently microbeads have become commercially available that only express a single HLA antigen (for example a specific bead within an assay set may express HLA-B7 and no other HLA specificity). This has increased the specificity of the assay markedly.

In addition to the fact that microbeads negate the need to constantly source fresh donor cells, they also allow the laboratory to monitor other HLA antibody specificities that may not be present on the graft. This helps to distinguish between a general upregulation of the immune response, such as that which may be associated with an inflammatory response, and a specific anti-graft response [116].

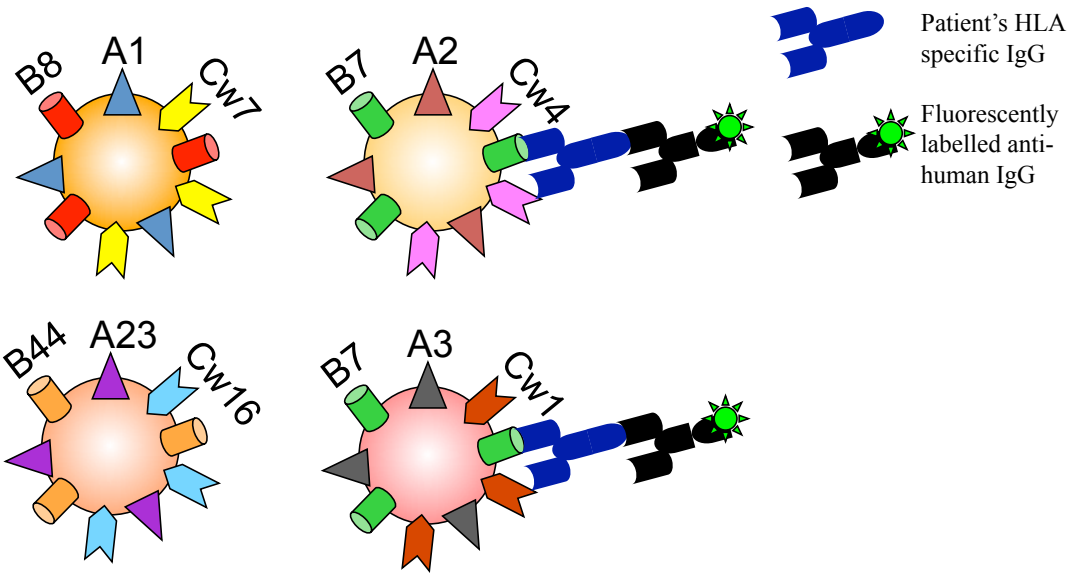


Figure 1.20: Principle of microbead analysis as detected on the Luminex platform. Fluorescently marked microbeads are coated with HLA protein. The beads are incubated with patient sera and HLA-specific antibodies are detected using labeled anti-human IgG.

Although the development of luminex-based technology has led to substantial progress in antibody detection compared with cell-based techniques, it soon became apparent that the assay did have technical issues, particularly when testing patients with high-titre antibodies. High-titre antibodies can exhibit a phenomenon known as the prozone effect, whereby high-titre antibodies can agglutinate in suspension and as a consequence can prevent themselves from binding to target antigen. This results in stronger antibody profiles within a patient sample being detected at reduced mean fluorescence intensity (MFI) levels, or in some cases not being detectable at all [117]. A similar effect whereby false low readout can be obtained in high titre samples is the high-dose hook effect [118]. This may cause inhibition of assay binding due to steric

hindrance and is investigated further in chapter 3. The presence of prozone, or the high dose hook effect, has to be overcome by serially testing sera. In addition to the obvious cost implications to repeat testing of patient sera, it is not always obvious when analyzing screening data when bead reactivities are being detected at false low levels. One potential solution has recently been suggested to overcome the apparent prozone effect is to add a small amount of ethylenediaminetetraacetic acid (EDTA) to the serum prior to testing [117]. EDTA has the ability to chelate metal ions, most notably calcium ions (Ca^{2+}). A suggested mechanism for the prozone effect is the binding of complement component C1 to the Fc portion of IgG1 and IgG3 and the addition of EDTA disrupts this Ca^{2+} dependent process. The presence of C1 bound to the HLA specific antibody is then thought to prevent the binding of the anti-human IgG labeled secondary detection antibody resulting in a lower detectable level of fluorescent marker. Further support for this hypothesis was gathered by addition of C1 inhibitor (C1INH) also leading to a marked reduction in the prozone effect.

Similarly, the presence of HLA-specific antibodies of IgM isotype have been put forward as a possible cause of reduced IgG binding in the luminex assay [119, 120]. It is hypothesized that the presence of HLA-specific antibodies of both IgM and IgG isotype within the same serum can lead to competitive inhibition and reduced binding of IgG antibodies. Therefore some laboratories recommend the routine treatment of all sera with DTT in order to reduce IgM binding capacity.

Modifications of the luminex assay have also been developed so that antibodies can be defined into those that fix complement and those that do not, with many of the early studies appearing to indicate that the presence of donor specific complement fixing anti-HLA antibodies is associated with increased risk of rejection and graft loss compared to non-complement fixing antibodies. Studies performed in renal transplant

and cardiac transplant setting has so far concluded that complement fixing antibodies confer increased risk of rejection [121-125].

Another technical issue surrounding the use of HLA antigen coated beads has recently emerged. During the manufacturing process there appears to be a significant proportion of HLA protein coupled to the microbeads that has been denatured to some extent. The presence of denatured antigen on the bead surface can lead to the presentation of a number of non-native HLA epitopes due to altered conformation of the protein. These novel epitopes appear able to bind to a number of common antibodies that may have arisen in response to a previous viral or bacterial infection. Literature is available describing the presence of HLA-specific antibodies in previously untransfused and untransplanted male volunteers as detected by microbead analysis. These antibodies were previously explained as the presence of 'naturally occurring' anti-HLA antibodies, but it now seems that a much more likely explanation is that these reaction patterns would be due to binding to non-native epitopes presented by a proportion of denatured antigen found on the microbead [79]. Strategies to identify reactions caused by denatured antigen binding have been described, with the most common being to acid treat the microbead set to fully denature the beads' protein repertoire. If it is suspected that all or part of an antibody profile is due to antibody binding to denatured protein, treating the bead set with an acidic buffer such as 100mM glycine pH2-3 will denature all the protein on the beads. If repeat testing with denatured beads gives the same antibody profile this confirms binding to disrupted HLA. Often, it is observed that the initial result is due to a combination of binding to both intact and denatured antigen, and that crucially antibody that recognizes non-native HLA epitopes are not to be considered clinically significant [126, 127].

1.11 HLA Incompatible (HLAi) Transplantation: Overview

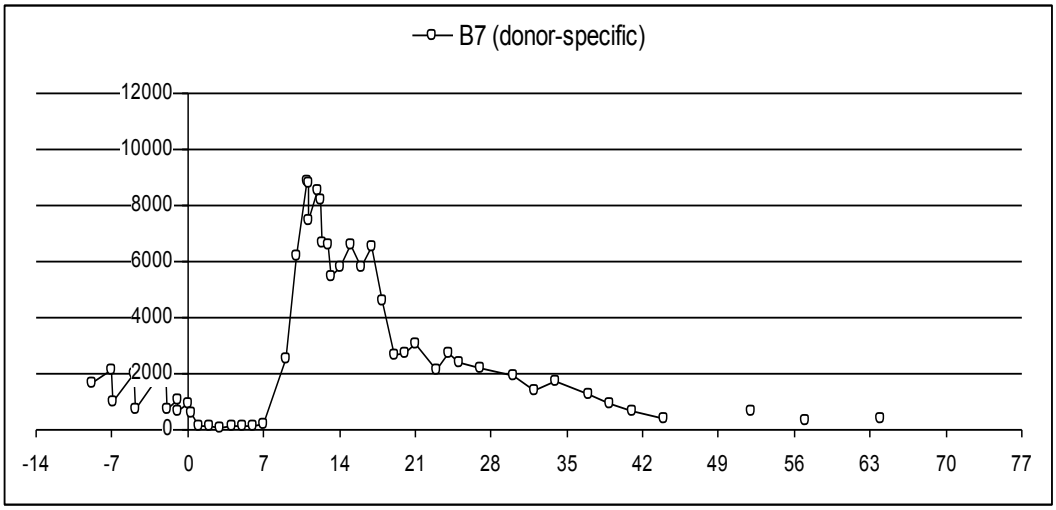
Early attempts to reduce the levels of HLA specific antibody in highly sensitised patients prior to transplantation were made in the mid 1980s. Taube et al described a series of 5 patients who were transplanted following reduction of their anti-HLA titres following a protocol of pre-transplant plasma exchange and prednisolone and cyclophosphamide based immunosuppression [128]. Reduction of antibody reactivity lead to successful transplantation in four of the five cases reported.

The Johns Hopkins University in the United States first developed a successful protocol for AIT in the late 1990s [129] which was a refinement of a number of earlier, less successful protocols [110, 128, 130]. This protocol involved the use of living donors which gave the added advantage that procedures could be planned well in advance providing an excellent opportunity to remove harmful antibody prior to transplant. Typically five sessions of plasma exchange were administered in the ten days leading up to the transplant with the aim being to reduce the level of DSA to that which would produce a negative crossmatch at time of transplantation. Interestingly this protocol also included the use of intravenous immunoglobulins (IVIg) on the basis that they may be able to block the action of any existing antibody and antibody that may resist plasma removal.

The last decade has provided a number of studies which have indicated that AIT can be successful, and that not all components of the early Johns Hopkins protocol are required. During the recent advancements in AIT centres have adopted different parts of the Johns Hopkins protocol, with good results being achieved by most centres [91, 105, 131].

The Local Experience

A protocol for pretransplant antibody removal using plasmapharesis has recently been developed at the University Hospital of Coventry and Warwickshire (UHCW) to overcome this barrier [91]. After nearly ten years, the results of the programme are proving to be very good [90, 107, 108, 116, 132]. One of the key features of the protocol is daily monitoring of DSA during the early post-transplant phase. This allows guided patient management, such as immunosuppression modification, as we have been able to recognise early signs of a rejection response in terms of rapid re-synthesis of prior DSA in the early post-transplant phase; we believe we are the only transplant centre in the world using this approach and have the resulting data and sample archive [133]. From over 100 procedures to date we are beginning to understand the predictive nature of the DSA response. Thus we have observed in certain cases, particularly those who undergo a rejection process, that after a rapid rise in DSA the antibody response is not sustained and following treatment and resolution of the rejection, the DSA is rapidly modulated [134]. Rejection in these cases has been successfully treated in all but one case, and the most effective treatment appears to be using the T-cell inhibitor OKT3 [90]. This whole process, from first rise in antibody, onset and resolution of rejection to antibody modulation takes about two-three weeks, as shown in figure 1.21. One further feature of this observation is the rapid loss of serum DSA which is far quicker than that predicted by the half-life of serum IgG; ie this is probably an active process. In contrast, with the absence of a rejection process following an early response there is usually sustained circulating antibody levels (figure 1.21).



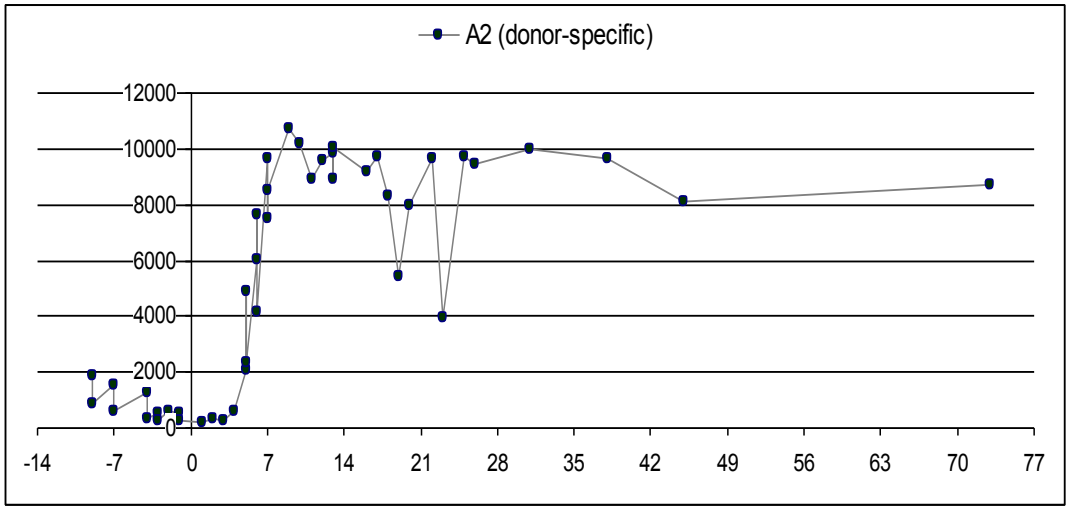


Figure 1.21: Daily monitoring of DSA levels- examples from our local cohort. The top graph shows DSA bead MFI levels in a patient who developed rejection at day 11. Pre-transpant levels show reduction following successive plasma exchange treatments. Post-transplant (day 0), DSA is seen to rise from day 7 and fall from day 10. The bottom graph shows DSA changes in a patient who did not experience a rejection episode despite a much more rapid onset rise in DSA levels followed by sustained levels of anti-donor antibody.

The daily monitoring of DSA in antibody incompatible transplantation has allowed us to observe three fundamental immunological phenomena; (1) an early humoral response which is damaging in some cases but not others, (2) antibody modulation as a result of the rejection process or its treatment, (3) active IgG removal from circulation. The identification of factors that may predict rejection is crucial, as rejection cannot be predicted based upon changes in DSA levels alone.

1.12 Hypothesis / aims

Organ transplantation has always been one of the major service areas of the Birmingham Histocompatibility and Immunogenetics (H&I) laboratory which has supported the West Midlands organ transplant programmes for over thirty years. The development and growth of renal transplantation in this region has always been accomplished by the laboratory working in partnership with the clinical units. Antibody incompatible transplantation requires a particularly close working relationship between laboratory and transplant unit in order to control the risks associated with this form of treatment and develop it further. The expansion of the programme of HLA incompatible transplantation nationwide requires the rapid development of UK H&I laboratories in order to provide a high level of service support to such schemes. At the University Hospital Coventry and Warwickshire NHS Trust programme we are leading the way in HLA antibody incompatible transplantation and have undertaken over 100 cases to date.

The project will use soluble HLA proteins (sHLA), produced via collaboration with Dr William Hildebrand at the University of Oklahoma, USA, to investigate the means by which the epitope specificity of anti-HLA antibodies can be determined using current screening methodologies. In addition the definition of exact epitope specificity

will facilitate attempts to quantify the exact concentration of HLA-specific antibody in the circulation, something which has hitherto not been achieved.

I will explore the potential influence of IgG subclass distribution on the pathogenicity of the anti-HLA response. Using our unique archive of daily samples from over 100 HLA incompatible transplants performed to date, I will investigate how the subclass specific IgG profile may alter through the course of a HLA incompatible transplant and assess whether any changes correlate with clinical outcome.

The project will also explore the possibility of developing a device to selectively remove HLA specific antibody from the patient prior to transplantation. Current technologies do not provide the specificity and often remove antibodies to other targets which may compromise patient immunity. Therefore we aim to develop a device in the form of a column containing HLA antigen bound onto a substrate, through which the patient's plasma will be passed and the HLA antibodies removed.

The competing solutions most frequently used are plasma exchange; double filtration plasmapheresis; immunoadsorption with columns containing polyclonal antibodies against human immunoglobulins and with columns containing staphylococcal protein A. These solutions have several shortcomings: (a) all are non-specific, removing antibodies against all antigens, rather than just against the donor kidney, resulting in increased infection rate, (b) effective removal of HLA antibodies by treatment of high volumes of plasma is not possible with plasma exchange and plasmapheresis, (c) double filtration plasmapheresis is frequently associated with complications such as fluid shift, hypotention and perioperative bleeding; d) in columns that remove just immunoglobulins, removal may be incomplete, especially in the case of Protein A, which does not remove IgG3 and IgM effectively. The competitive advantage will be specificity, tolerability, safety and effectiveness of the proposed columns containing HLA antigen. The device is designed to remove only antibodies

against HLA, leaving the remaining humoral immunity intact. The device will be well tolerated, reducing the likelihood of fluid shifts, hypotension and perioperative bleeding, as one would expect from experience with the Glycorex immunoadsorption columns for blood group antibodies. This allows larger volumes of plasma to be treated than possible with conventional plasmapheresis. This will both make HLA antibody incompatible treatments more effective and safer, opening kidney transplantation to patients currently ineligible for antibody incompatible transplantation, such as those without living donors, restricted to waiting for a deceased donor transplant, and those with significant comorbidity factors.

The ability to isolate HLA-specific antibody from patient serum using a HLA protein column will provide a novel means with which to study these antibodies. Purified antibody preparations will be used to investigate the epitope specific nature of antibody binding, and also to define the factors that may be involved in determining the functional capacity of these antibodies, and the implications upon graft survival.

Chapter 2

METHODS

2.1 BACKGROUND

This project used a range of standard protein chemistry techniques to purify proteins for further analysis. The following sections describe all of the techniques used in detail. In addition, the project employs a range of standard assays used in the Birmingham Histocompatibility and Immunogenetics laboratory, the techniques given here represent those used in the clinical laboratory.

All commercially available kit-based assays were performed as according to manufacturers' recommendations, with the exception of microbead assays for HLA-specific antibody determination which were modified to use reduced reagent volumes in order to abrogate the high costs of the assays.

Blood samples and other human fluids used in this thesis were obtained from patients undergoing HLA antibody-incompatible renal transplantation at the University Hospital Coventry & Warwickshire. An informed consent was obtained from all patients and the study met with local ethics committee approval (Coventry Local Research Ethics Committee ref 055/10/03).

2.2 HLA PROTEIN PRODUCTION AND PURIFICATION

The use of highly purified soluble HLA (sHLA) proteins form the basis of many of the methodologies used in this thesis. The two production methods which were used are described in detail below. These methodologies were kindly provided by Professor Paul Moss from the University of Birmingham [135] and Dr William Hildebrand from the University of Oklahoma in collaboration with the private biotechnology company Pure Protein LLC (Oklahoma City, USA)[136].

Where sHLA is used to generate data, the exact source of the protein will be indicated in the relevant methods section.

2.3 SOLUBLE HLA (sHLA) PROTEIN PRODUCTION AND PURIFICATION: BIRMINGHAM PROTOCOL

All buffers used are listed in Table 2.1:

2.3.1 Expression of sHLA (MHC class1 heavy chain and β_2M in E.Coli)

To make competent cells a single E.Coli colony was used to inoculate 5ml Luria Broth (LB) and then agitated at 37°C overnight. The cells were then transferred into 200ml LB and grown at 37°C until the OD_{600} = 0.48. The OD_{600} reading is a measure of bacterial growth within a suspension. The preparation was then incubated on ice for 15 minutes. Preparations were then centrifuged at 6000 rpm for 10 minutes at 4°C then re-suspended in TFB1 buffer and incubated on ice for one hour. Preparations were centrifuged once more at 6000 rpm for 10 minutes then re-suspended in 8ml TFB2 and incubated on ice for one hour. Samples were then distributed into 100-200µl fractions and stored at -70°C.

Table 2.1: Production of sHLA. The full list of buffers used in the production of sHLA protein production and purification according to the University of Birmingham protocol.

Reagent	Composition
Luria Broth (LB)	10g/l Trytone 5g/l Yeast 10g/l Sodium Chloride
TFB1 Buffer pH to 5.8	30mM Potassium Acetate 100mM Rubidium Chloride 10mM Calcium Chloride 50mM Manganese Chloride 15% (v/v) Glycerol
TFB2 Buffer pH to 6.5	25mM Calcium Chloride 10mM Rubidium Chloride 15% (v/v) Glycerol
Agar-LB Plates (500ml)	5g Tryptone 5s Sodium Chloride 2.5g Yeast 0.02mg/ml Ampicillin
Triton Wash	0.5% Triton X-100 50mM Tris pH 8.0 100mM Sodium Chloride 0.1% Sodium Azide 1mM EDTA 1mM DTT
Re-suspension Buffer	50mM Tris pH 8.0 100mM Sodium Chloride 1mM EDTA 1mM DTT
Urea Solubilisation Buffer	8M Urea 50mM MES pH6.5 0.1mM EDTA 0.1mM DTT
Refolding Buffer	Tris pH 8.0 400mM L-Arginine 2mM EDTA 5mM reduced glutathione 0.5mM oxidised glutathione 0.1mM PMSF

Transformation of Competent Cells

To transform competent cells, 1-2μl of plasmid were added to 100-200μl of competent cells (use plasmid PGM17 for HLA-B*07:02 and PET23 for HLA-A*0201, as kindly provided by Professor Moss) and left on ice for one hour. Samples were then heat shocked in a water bath set to 42°C for exactly 45 seconds. Following the addition of 200μl LB, cells were shaken and incubated at 37°C for 1 hour. 200μl of cell preparation were put onto agar plates containing 0.02mg/ml Ampicillin and incubated at 37°C overnight.

2.3.3 Expression of MHC Class 1 and β_2M.

Sufficient LB was prepared and autoclaved in advance (typically four 2 litre flasks). Overnight cultures of transformed bacteria were prepared by pouring out 2 x 40ml LB and adding 40μl Ampicillin stock (100mg/ml) and 1 colony from the transformed agar plate to each 40ml aliquot. This was done in duplicate in the event that one tube fails to grow. Incubated, shaking, at 37°C overnight.

Subsequently, 1ml Ampicillin (100mg/ml) stock was added to each litre flask of media, 1ml of media was taken as a blank control for the spectrometer, and 10ml of overnight culture was added to each flask. Samples were incubated, shaking at 37°C for two hours until the OD_{600} = 0.3 – 0.6. Next, 0.5ml was taken for the pre induction sample protein gel analysis (see section 2.1.3 for protein gel protocol). Protein induction was instigated by adding 0.5ml 1M isopropyl-beta-D-thiogalactopyranoside (IPTG) to each flask (IPTG is a synthetic analogue of lactose which induces expression of cloned genes under the control of the *lac* operon), followed by incubation for a further 4-5 hours. Another 0.5ml sample was taken for the gel, post induction sample (Figure 2.1).

The bacteria were then transferred into plastic containers and centrifuged in a pre-cooled centrifuge at 4000 rpm for 20 minutes. The supernatant was discarded and the pellet was re-suspended in 2ml cold PBS. The bacteria were kept on ice and transferred to a 50ml centrifuge tube. The container was rinsed with a further 2ml cold PBS. The bacteria were stored at -70°C at this stage until required.

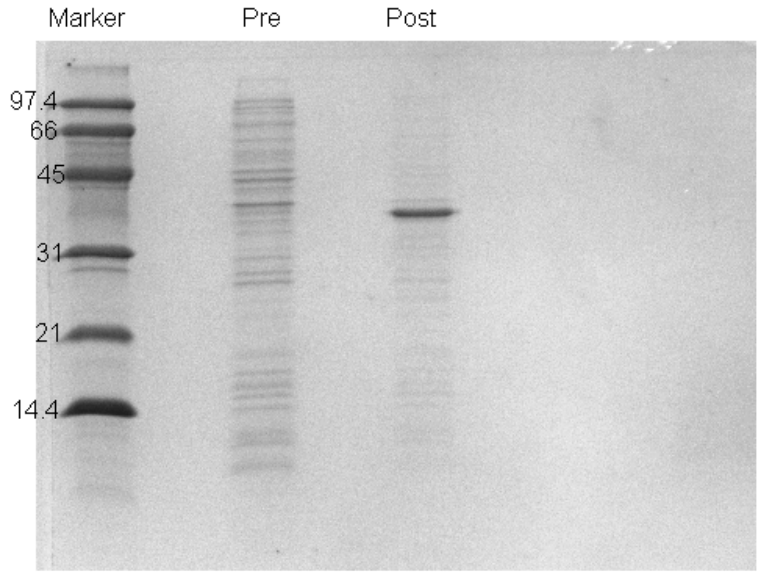


Figure 2.1: Protein gel image showing addition of IPTG has induced bacteria to produce MHC heavy chain. The pre-induction sample shows no protein band, whereas post-IPTG induction a protein band of approximately 40kDa is clearly visible and corresponds to induction of MHC class I heavy chain production.

2.3.4 Purification of Protein Containing MHC Class I from Inclusion Bodies.

The bacteria were allowed to thaw for a couple of hours at room temperature, and once thawed were kept on ice. Triton wash and Re-suspension buffer were prepared and placed on ice. The bacteria were then transferred to a glass beaker and placed on

ice. More PBS (up to 50% v/v) was added if required – this enabled more rapid sonication. Sonication was performed on ice, as it is vital the sample should not overheat. The next step was to add 0.1mM protease inhibitor (PMSF) and lysozyme (1.075ml of 10mg/ml per litre of bacteria) and incubated on ice for 30 minutes or until the sample had a gloopy consistency. Using the appropriate titanium horn, the bacteria were sonicated in bursts of 45 seconds, followed by 1 minute (between every 2 bursts) on ice to allow the sample to cool down. This was repeated at least 5 times until the sample was completely lysed. The sample was stored on ice again for 30 minutes to allow the lysozyme to completely breakdown any non-protein material.

The sonicated sample was then spun in a pre-cooled (4°C) centrifuge at 15000 rpm for 10 minutes. The supernatant was discarded and 4ml per litre of Triton wash was added. The sample was transferred into a glass homogeniser using a disposable loop or re-suspended with a pastette and transferred to the homogeniser. The sample was homogenised until it was completely re-suspended then transferred into plastic containers and centrifuged at 15000 rpm for 10 minutes at 4°C. The supernatant was removed and this procedure was repeated twice i.e. a total of 3 washes. Whilst washing, if a green/brown scum was seen on the top of the pellet this was removed and discarded. After the final wash the supernatant was discarded and 2ml per litre of re-suspension buffer was added. The sample was transferred into a clean glass homogeniser and spun at 15000 rpm for 10 minutes at 4°C. The supernatant was discarded and the wash step was repeated.

2.3.5 Urea Solubilisation of Proteins Containing MHC Class 1

To the pellet, 20ml (for 4 litres of bacteria) of freshly prepared Urea solution was added and re-suspended by vortexing. The pellet was incubated at 4°C overnight, rotating to allow the protein to solubilise completely. MHC class I heavy chain protein samples were centrifuged at 15000 rpm for 5 minutes at 4°C to remove any insoluble material. The protein was now suspended in the supernatant. The protein concentration was measured and dispersed into aliquots of 10mg/ml fractions in Eppendorf tubes and stored at -70°C. The protein should be stable for up to a year.

2.3.6 Refolding MHC Class 1 and β_2M in vitro

Prior to refolding, 1 litre of the refolding buffer was prepared (table 2.1) and allowed to cool to 4°C for 1 hour, whilst being constantly stirred. Once chilled, the pH was adjusted to 8.3 and 1ml of 0.1M PMSF was added prior to the addition of the protein. 5mg peptide of choice (e.g. NLV, VTE) dissolved in 500ul DMSO, was then added to the refolding buffer. 10mg of β_2M was thawed and transferred to a 50ml tube. Slowly, the refold buffer was added to the 50ml tube drop-wise. A total of 10-15 ml refold buffer was added slowly to avoid protein precipitation. Finally this mixture was added slowly into the 1 litre refold buffer with constant stirring allowing the β_2M to refold in the absence of heavy chain for 30 minutes. One quarter of the total amount of heavy chain required (7.5mg at this stage) was thawed and diluted in 5M urea (to a concentration of 1mg/ml) and transferred to a 50ml tube. Slowly, the refold buffer was added to the 50ml tube drop-wise. After adding a total of 10-15 ml refold buffer this

mixture was slowly incorporated into the 1 litre refold buffer with constant stirring, as before.

The mixture was then left to refold overnight, with constant stirring. The refold was repulsed with heavy chain only using the method employed for previous additions. The refold was pulsed up to 4 times in a period of 24 hours until all 30mg were added.

2.3.7 Concentration of Refolded MHC Class I Complexes.

Before concentration, the refold was passed through 0.45uM filter to remove any aggregates. Next the refold mixture was added to a Millipore Amicon stirred cell Model 8400 filter, fitted with a 10,000 MW polyethersulfone (PES) membrane and concentrated under 50psi pressure with Nitrogen gas. The procedure was repeated until all the refold mixture was concentrated. The refolded protein was captured on the membrane so it was not allowed to dry out. The refold was concentrated from 1 litre to 5-10ml and filtered through a 0.45 syringe filter prior to buffer exchange (see protein concentration and buffer exchange protocol in section 2.5.2).

2.4 SOLUBLE HLA (sHLA) PROTEIN PRODUCTION AND PURIFICATION: OKLAHOMA PROTOCOL

The following protocol is used to create soluble class I and II HLA proteins and is in routine use in the research laboratory of Dr William Hildebrand at the University of Oklahoma. The proteins are also produced commercially by this same method by Pure Protein LLL (Oklahoma City USA). I received training and experience of the technique described here whilst on secondment to the University of Oklahoma and Pure Protein LLC in April 2011. The majority of the proteins produced for use in this project were supplied directly by Pure Protein LLC under the terms of an official collaborative agreement between University of Oklahoma, Pure Protein LLC, University of Warwick, and University Hospital Coventry and Warwickshire NHS Trust.

2.4.1 Soluble Class I HLA Protein Production

To produce secreted HLA Class I, α-chain cDNA of HLA-A, B, or C was modified at the 3' end by PCR mutagenesis to delete codons encoding the transmembrane and cytoplasmic domains and to add the VLDLr purification epitope (Figure 2.2).

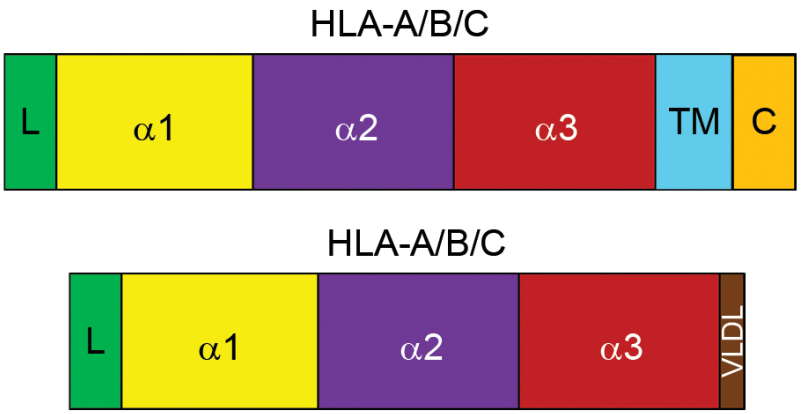


Figure 2.2: Schematic representation of the cDNA use to produce the α-chain domains of secreted HLA class I. The codons encoding the transmembrane (TM), and cytoplasmic domains (C) were deleted, and a VLDL expression tag was incorporated.

The resulting construct was cloned into the mammalian expression vector pcDNA3.1(-) that contained a geneticin resistance cassette. Class I HLA deficient human cell line 721.221 [137] was transfected with this expression construct and drug resistant clones were selected in growth media containing G418, an aminoglycoside antibiotic [138], at a concentration of 0.8mg/ml (see figure 2.3A), in addition β2m protein was added from glycerol stock at a concentration of 0.5mg/ml. sHLA production was measured using a capture ELISA where W6/32 was the capture antibody and anti-β2m was the detecting antibody. sHLA producing clones were sub-cloned into 96 well plates by limiting dilution and high producing sub-clones were expanded and seeded in AccuSyst-Maximizer hollow fibre bioreactor (Biovest International, Tampa, USA).

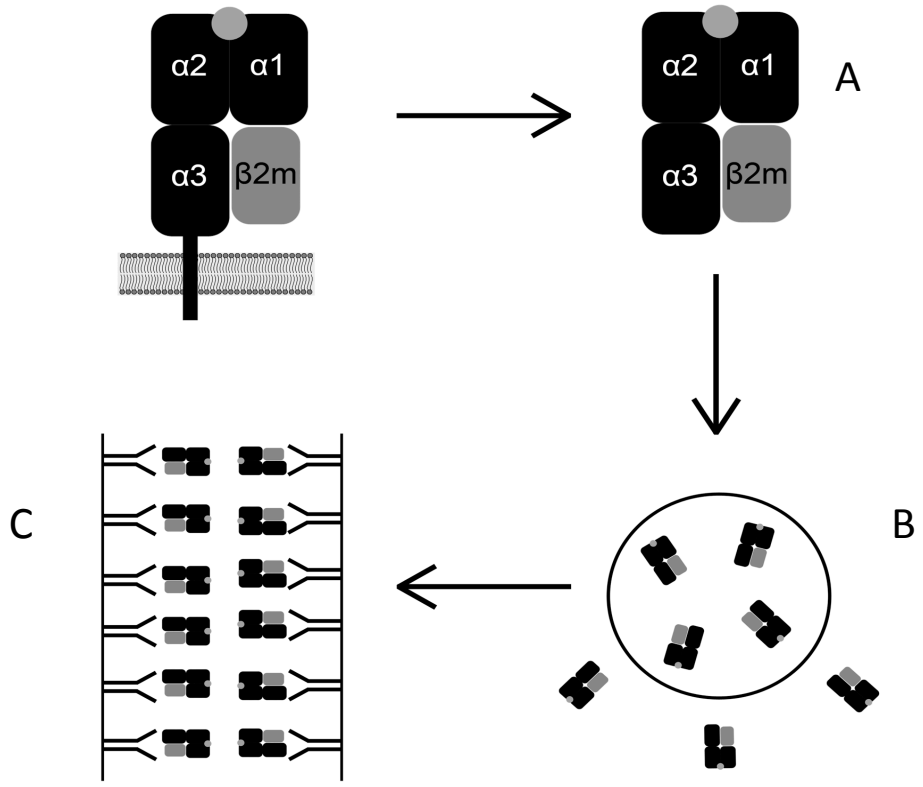


Figure 2.3: Overview of sHLA class I production. A) HLA constructs are first cloned into HLA class I deficient human cell-line 721.221. B) High sHLA producing clones were seeded into large hollow fibre bioreactors and the secreted sHLA was hervested. C) sHLA is purified by passage through a W6/32 affinity chromatography column.

Approximately 500 mg of sHLA was harvested from the sub-clones on the bioreactor (figure 2.3B). Supernatant containing sHLA was loaded on a W6/32 immunoafffinity column and washed with 40 column volumes of 20mM phosphate buffer pH 7.4. Class I sHLA molecules were eluted from the affinity column with 50mM DEA at pH 11.3, neutralized with 1M TRIS pH 7.0, and buffer exchanged and stored at 1 mg/ml in sterile PBS (figure 2.3C).

2.4.2 Class II sHLA Protein Production.

To produce secreted Class II sHLA molecules, α-chain cDNAs of HLA-DRA1, DQA1, DPA1 and HLA-DRB1,DRB3,DRB4,DRB5,DQB1,DPB1 were modified at the 3' end by PCR mutagenesis to delete codons encoding the transmembrane and cytoplasmic domains and to add the leucine zipper domains. For DRA1,DQA1 and DPA1 a 7 amino acid linker (DVGGGGG) followed by leucine zipper ACIDp1 was added. For DRB1, DQB1, DPB1 the same linker was used, followed by leucine zipper BASEp1 sequence. The addition of the leucine zipper enables the α and β chains of the class II molecule to maintain native conformation (Figure 2.4). The HLA class II molecule exists as an alpha/beta heterodimer and replacing the transmembrane and cytoplasmic domains with the complemenatary leucine zipper domains ACIDp1 and BASEp1 produces the correct conformational structure.

The class II sHLA-DRA1, DPA1, DQA1 was cloned into the mammalian expression vector pcDNA3.1(-) (Geneticin, Invitrogen, CA, USA) and sHLA-DRB1, DQB1, DPB1 was cloned into pcDNA3.1(-) (Zeocin, Invitrogen, CA, USA). The HLA class II deficient mouse B-LCL cell line NS1 (ATCC # TIB-18) was transfected by electroporation simultaneously with sHLA-DRB1 and DRA1, DQB1, and DQA1, DPB1 and DPA1.

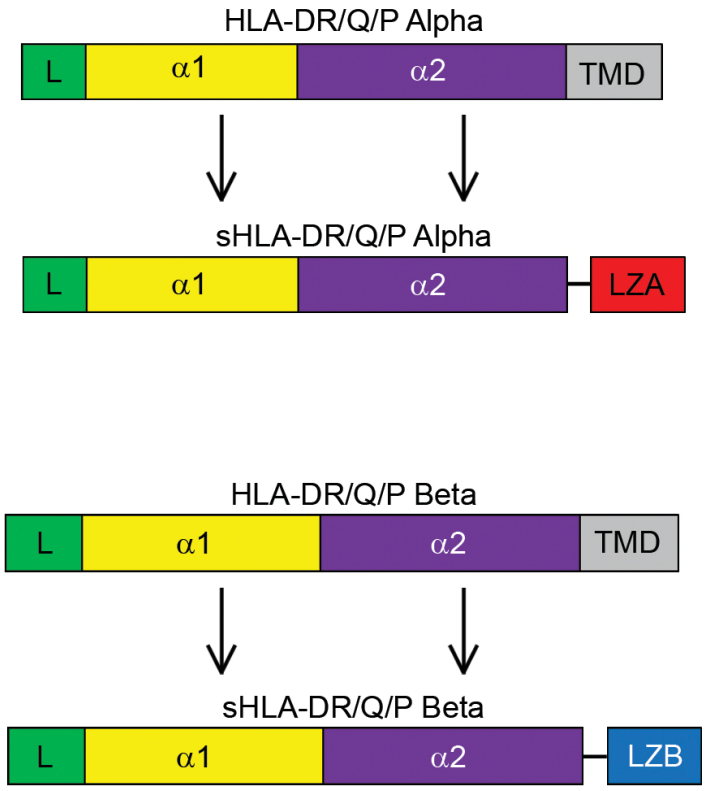


Figure 2.4: Diagrammatic representation of the cDNA constructs used to produce the HLA class II proteins. The transmembrane domains (TMD) were removed and two complementary leucine zipper domains (LZA, LZB) were added to each chain to maintain the structural stability of the sHLA class II protein.

Two days post-electroporation, cells were transferred into selective growth media containing antibiotics G418 (0.8 mg / ml) and zeocin (1mg/ml). Drug resistant stable transfectants were tested for production of sHLA class II molecules by sandwich ELISA using L243 (Leinco Technologies, St Louis, USA) as a capture and a biotinylated anti-HLA class II commercial antibody for detection (Product number FH0002, One Lambda Inc, CA, USA). Positive cells were sub-cloned into 96 well plates by limiting dilution. Individual wells with clonal cell populations were tested for

the production of sHLA class II by ELISA and high producers were expanded in an AccuSyst-Maximizer hollow fiber bioreactor (Biovest International, Tampa, USA). A saturating capacity of approximately 200 mg of sHLA-DR11 was harvested from each bioreactor. sHLA-DR/DP/DQ containing supernatant was loaded onto L243 or anti-leucine zipper immuno-afffinity columns and washed with 40 column volumes of 20mM phosphate buffer pH 7.4. Class II sHLA molecules were eluted from the affinity column with 50mM DEA at pH 11.3, neutralized with 1M TRIS pH 7.0, and buffer exchanged and stored at 1 mg/ml in sterile PBS.

2.4.3 sHLA Purity Analysis

The purity of the sHLA complexes were assessed and analysed using mass spectroscopy (MS). Ten microgrammes of sHLA was reconstituted in 30µl 25mM ammonium bicarbonate in ultrapure water. The protein samples were then reduced by the addition of 1.5µl of 100mM DL-Dithiothreitol (Sigma-Aldrich, UK), to give a final concentration of 5mM. The samples were immediately incubated at 95°C for five minutes to allow sample reduction. Proteins were then alkylated by adding 3µl of 100mM iodoacetamide (Thermo-Scientific, Loughborough, UK) to give a final concentration of 10mM followed by incubating in the dark for 1 hour at room temperature. A two-step protein digestion was then performed by first adding 1µl of 100ng/µl MS grade L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin (Thermo Scientific, Loughborough, UK) for a 1:100 w/w enzyme protein ratio followed by incubation at 37°C for three hours. The same amount of trypsin was then added again to adjust the w/w enzyme/protein ratio to 1:50 and the samples were

incubated at 37°C overnight. The protein digestion was then stopped by adding 20µl 1% trifluoroacetic acid (TFA) followed by drying in the speed-vac (Eppendorf, UK) without heat. The digested tryptic peptides were then reconstituted in 30µl of 30% acetic acid / 70% ultra-pure water and 5µl was then loaded onto the UltiMate 3000 HPLC system (Dionex, CA, USA) using a PepMap100 C18 75µm x 15cm, 3µm 100Å reverse phase column. Peptides were eluted using H_2O/acetonitrile (20:80v/v), 0.08 formic acid as the solvent at the following gradient: 2% for 4 minutes, 2-10% for 1 minute, 10-35% for 45 minutes, 35-70% for 10 minutes, 70-80% for 3 minutes, hold at 80% for 2 minutes, then 80% reducing to 2% in 2.5 minutes. The gradient was then held at 2% for 20 minutes. Peptides were analysed on a QTOF Qstar Elite mass spectrometer (ABI MDS Sciex, Framingham, USA) and analysed with Mascot software. Figures 2.5 and 2.6 show the MS analysis of sHLA-A2 and DR11 constructs.

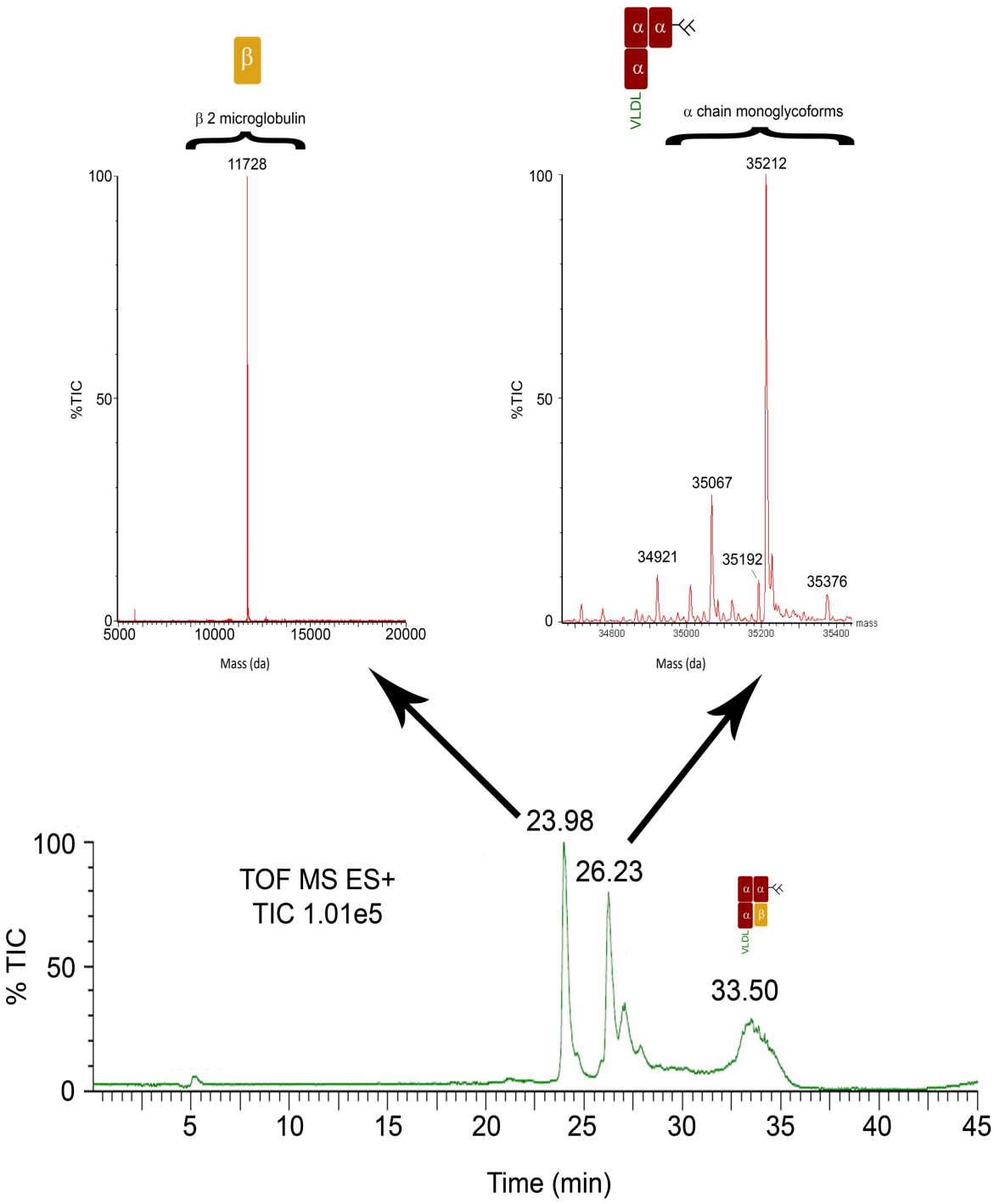


Figure 2.5: Mass spectroscopy analysis of sHLA-A2 proteins. Top panels represent a close-up view of the bottom panel broad purity analysis and show the minimal protein contamination in either the β-2 microgolublin or α-chain fractions.

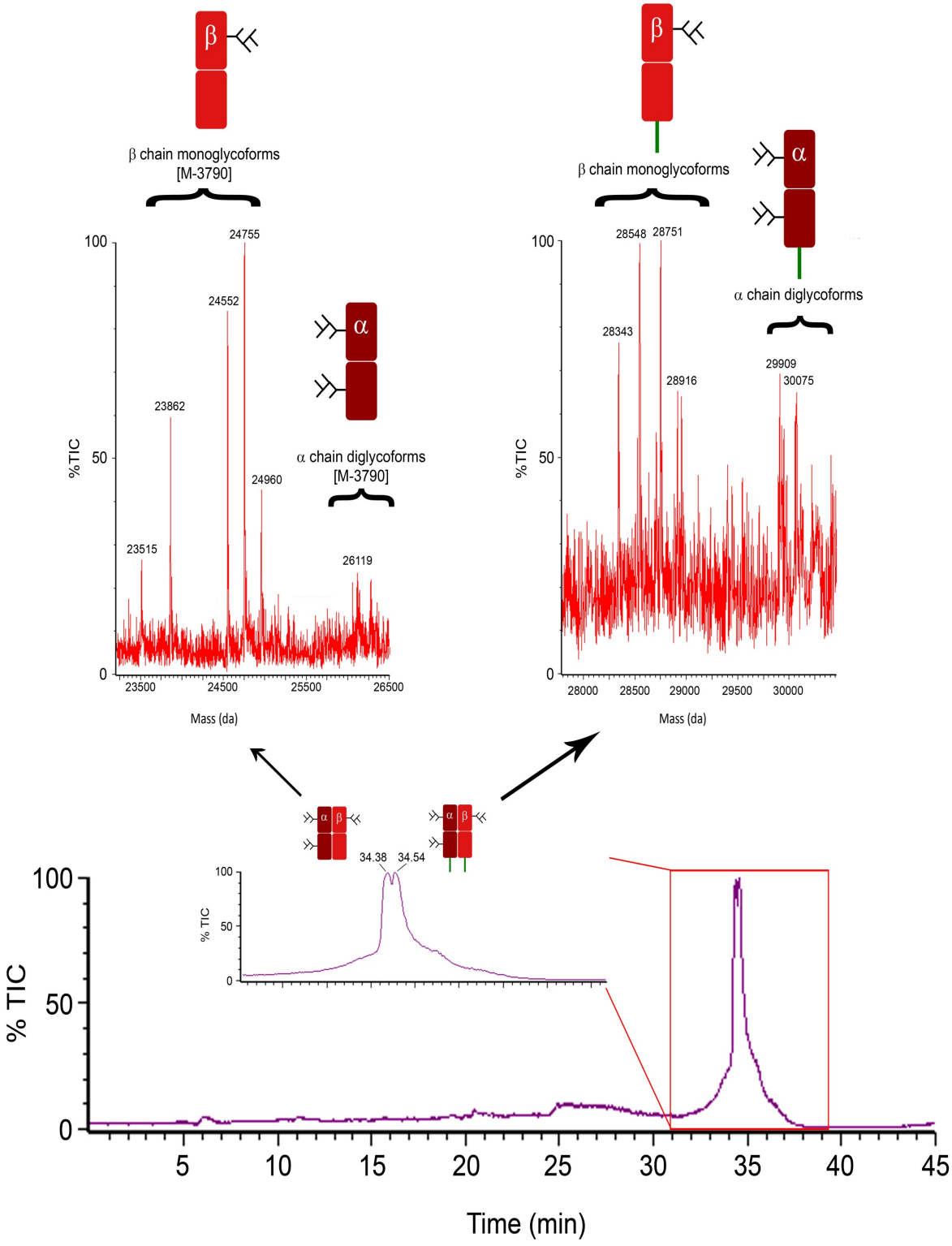


Figure 2.6: Mass spectroscopy analysis of sHLA-DR11 proteins. Top panels represent a close-up view of the bottom panel broad purity analysis. Glycoforms of both the DRB α and β chain show minimal protein contamination.

2.5 PROTEIN CHEMISTRY TECHNIQUES

The following section describes the techniques used to handle proteins for use in various sections of the study. The ability to correctly identify, analyse, and quantify specific protein preparations underpins many of the practical elements of the study. Where applicable the names of kit manufacturer's are given and the detailed composition of proprietary buffers has also been provided.

Values given to represent protein concentration will have been determined by either the BCA assay method, or by direct nanodrop OD_{280} measurement. This will be stated in the text where required. In some instances where both techniques have been used to quantify a specific protein concentration, a composite concentration value will be given which will represent the mean protein concentration derived from the two techniques.

2.5.1 Protein Gel Electropheresis.

This technique uses SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) to separate proteins according to their size, and no other physical feature. SDS denatures the proteins into a linear conformation and coats the protein with a negative charge [139].

Prior to protein gel electropheresis sample buffer stock for reducing or non-reducing conditions was prepared as required to the recipe given in table 2.2. Generally, 20µl each sample was added to 20µl loading buffer. If reducing conditions were required, for example to break down the IgG molecule into its constituent heavy and

light chain components, samples were incubated at 60°C for 10 minutes following addition of reducing agent as outlined in table 2.2.

Table 2.2: Sample buffer stock preparation for protein gel electrophoresis. Protocols for running samples in both reducing and non-reducing conditions are given.

Sample Buffer Stock	Reducing	Non-Reducing
4X NuPage LDS sample buffer (Invitrogen CA, USA) (70% Glycerol, 10% Lithium dodecyl sulfate	200ul	280ul
ddH20	120ul	200ul
10X NuPage Sample Reducing Agent (Invitrogen CA, USA) (500mM dithiothreitol)	80ul	0ul

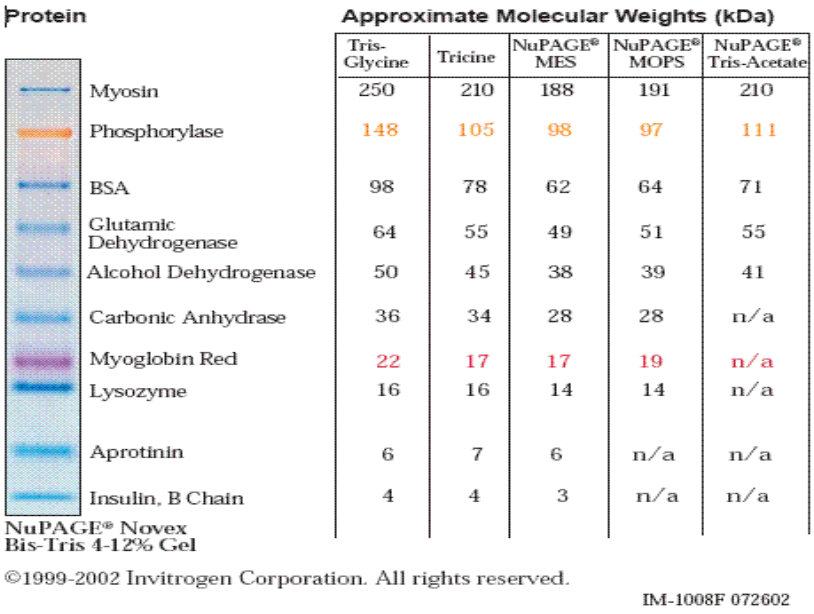
NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen, CA, USA) was removed from the packaging and the adhesive strip was removed to expose the electrode. Holding the sides of the gel cartridge firmly, the comb that protects the wells of the gel was carefully removed taking care not to damage the wells. Preservative was thoroughly rinsed from the wells with ddH_20. The gel was placed in the central chamber of the electropheresis chamber and locked into place. The gel chamber was filled with either MES or MOPS buffer as detailed in table 2.3 ensuring that the top portion of the gel was fully submerged. MES and MOPS buffer can be used interchangeably distinguished by a slight difference in the alkaline pH of each buffer.

Table 2.3: Proprietary buffer preparations used to prepare the gel tank for electrophoresis.

MES Buffer	MOPS Buffer
30ml 20X MES SDS (Invitrogen CA, USA) (5% Sodiumdodecyl Sulphate) 570ml ddH20	30ml 20X MOPS (Invitrogen CA, USA) (20% 4-Morpholinepropanesulfonic acid, 10% TRIS Base, 5% sodium dodecyl sulfate) 500ml ddH20

Samples were then loaded into the wells of the gel. In lane 1 of the gel 5µl of appropriate protein size ladder was loaded to assess size of protein product in sample well. The most commonly used protein size marker ladder was the Seeblue plus2 protein standard (Invitrogen CA, USA), which consists of 10 pre-stained protein bands in the size range 4-250kDa (figure 2.8). 20µl of sample/loading buffer mixture was loaded into the other wells of the gel as required. The lid was then placed on the gel tank and electropheresis was performed at 200V for 35 minutes.

Following electropheresis the Bis-Tris gel was removed from the gel cartridge and placed into a gel-washing basket. The gel was then submerged in double-deionsied water (ddH_20) and microwaved at full power (850W) for 10 seconds. The ddH_20 was then exchanged for fresh ddH_20 and the wash procedure was repeated twice more. The gel was then submerged in Coomassie blue stain (Invitrogen CA, USA) and placed on a rotary stirrer until protein bands were clearly visible.



Protein	Approximate Molecular Weights (kDa)				
	Tris-Glycine	Tricine	NuPAGE® MES	NuPAGE® MOPS	NuPAGE® Tris-Acetate
Myosin	250	210	188	191	210
Phosphorylase	148	105	98	97	111
BSA	98	78	62	64	71
Glutamic Dehydrogenase	64	55	49	51	55
Alcohol Dehydrogenase	50	45	38	39	41
Carbonic Anhydrase	36	34	28	28	n/a
Myoglobin Red	22	17	17	19	n/a
Lysozyme	16	16	14	14	n/a
Aprotinin	6	7	6	n/a	n/a
Insulin, B Chain	4	4	3	n/a	n/a

NuPAGE® Novex
Bis-Tris 4-12% Gel

IM-1008F 072602

Figure 2.9: Invitrogen Seeblue Plus2 protein marker. A range of protein markers of various sizes are provided with the approximate molecular weight values given for a range of sample buffers including MES and MOPS.

2.5.2 Protein Concentration and Buffer Exchange

This project relies heavily upon the production of purified sHLA proteins and HLA-specific antibodies selectively isolated from patient samples (see sections 2.8.3 and 2.8.4). The protocols required to isolate these proteins often leads to a protein eluate that is high in buffer volume and consequently the concentration of the desired protein is often low. In addition the buffers required to elute specific proteins are often of extreme pH (acidic or alkaline depending on the nature of the protein being eluted), and unsuitable for long-term protein stability. Therefore it is important to have a robust

protocol in place to be able to concentrate the protein down to a usable concentration, whilst at the same time exchanging the elution buffer for a more physiologically stable storage buffer, such as phosphate buffered saline (PBS) pH7.

Soluble HLA (sHLA) protein and HLA-specific antibody preparations were concentrated and buffer exchanged using 3000 molecular weight cut-off (MWCO) 20ml centrifugal spin concentrators (Vivaspin 20, Sartorius Stedim, UK).

To prepare the concentrator, 15ml PBS pH7.4 was added to the concentrator then centrifuged at 3000g for 30 minutes. The filtrate was then discarded and the concentrator was topped up with up to 15ml of protein sample solution then centrifuged again at 3000g for 30 minutes. The filtrate was then saved into an appropriately labelled 500ml bottle and stored to guard against loss of protein at this stage. The entrapped concentrate was topped up with 15ml fresh PBS pH7.4 and centrifuged at 3000g for 45 minutes (figure 2.10).

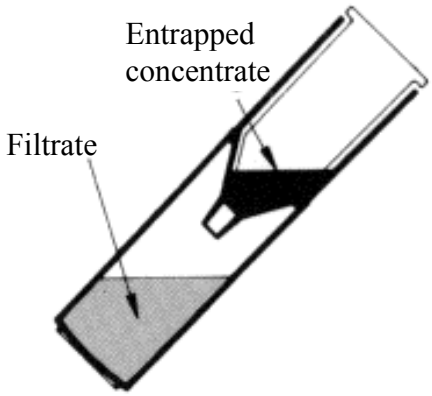


Figure 2.10: Protein concentration using a 3000 MWCO spin concentrator. Proteins larger then 3000MW are retained in the upper filter capsule, whilst smaller components pass through and are collected in the lower chamber.

The procedure was then repeated three more times so that complete buffer exchange can occur. The entrapped concentrate was then carefully removed using a fine tipped pipette. The concentrator was flushed with 200µl fresh PBS pH7.4 and this was combined with the concentrated protein sample. The sample was then prepared for storage at 4°C by passing through a 0.2µM filter and stored directly in sterile screw cap tubes.

2.5.3 Protein Quantification using BCA Assay

Bicinchoninic acid (BCA) protein assay kit was used to determine the quantitation of total protein (Thermo Scientific, Loughborough, UK). The BCA assay relies on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. Via a two-step process in which protein is chelated with copper, BCA invokes a colour change upon reaction with the reduced (cuprous) cation that was formed in step one [140].

Protein concentrations are generally determined in reference to standard dilutions of a common reference protein such as bovine serum albumin (BSA). Addition of BCA assay working reagent provides a colourimetric reaction, the intensity of which is detected by absorbance at 562nm and displays a near linear relationship with increasing protein concentrations.

Albumin BSA standards were prepared in the range 0-2000µg/ml by dilution of stock concentration with PBS pH7.4. Test samples were prepared at neat and 1 in 10 dilution and 100µl of each sample and diluted standard was added to disposable cuvettes. Each sample was set up in triplicate. All test and standard samples were then

incubated at 22°C for 2 hours. Samples were tested for absorbance at 562nm using a Perkin Elmer Lambda 2 spectrophotometer (Perkin Elmer, Waltham, USA). A cuvette filled with ddH_20 was used to zero the instrument. The 562nm absorbance value of the blank BSA reference was subtracted from the 562nm absorbance reading of all other individual standard and test sample replicates. The mean 562nm absorbance of the diluted standard triplicates were plotted to produce a standard curve (Figure 2.11). Protein concentrations of test samples were determined by comparing the mean of the test sample triplicates against the standard curve.

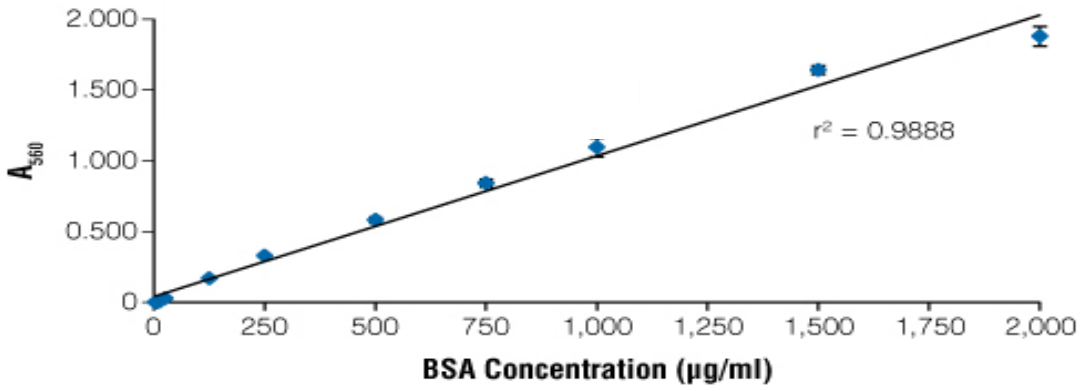


Figure 2.11: Colour response standard curve for bovine serum albumin (BSA) prepared for determination of protein concentration using the BCA assay (Thermo Scientific, Loughborough, UK). Absorbances of test samples at 562nm can be read directly off the standard curve to derive a protein concentration value.

2.5.4 Protein Quantification Using Nanodrop

The NanoDrop 1000 spectrophotometer (Thermo Scientific, Loughborough, UK) will accurately measure protein samples in the range of 0.1-100mg/ml. The protein A280 function was used to measure absorbance at 280nm of purified protein. Proteins that contain tryptophan (Trp), Tyrosine (Tyr), or cys-cys disulphide bonds will absorb in the ultra-violet (UV) range (280nm is indicative). Based on the absorbance values of these residues it is possible to extrapolate and give an accurate estimation of total protein content within a sample [141].

To assess protein concentration 2µl ddH_20 was pipetted on to the sample pedestal to initialise the NanoDrop. A blank measurement was then taken using 2µl PBS pH7.4. Protein concentration of test samples was then determined by subtracting blank absorbance from the measured absorbance at 280nm of each sample.

2.6 HISTOCOMPATIBILITY AND IMMUNOGENETICS TECHNIQUES

The following section describes various techniques used in the Histocompatibility and Immunogenetics department at NHSBT Birmingham. The evolution and clinical relevance of these techniques have been discussed in the introduction section, this section describes how the tests are performed and interpreted in greater detail.

2.6.1 Isolation of Lymphocytes from Peripheral Blood

Lymphocytes are isolated from human peripheral blood samples using density gradient separation medium (Lympholyte-H, Cedarlane Labs, Burlington, Canada). The cell population resulting from the following protocol is enriched for viable human lymphocytes and monocytes [142]. The use of purified lymphocyte populations is integral to accurate cytotoxic and flow cytometry crossmatch procedures.

Human blood was collected in a suitable tube containing anticoagulant (usually EDTA) and diluted 50:50 with cell culture medium (RPMI 1640, Gibco, UK). Diluted blood was then layered slowly over Lympholyte-H in a 2:1 ratio i.e 14ml diluted blood layered over 7ml Lympholyte-H, keeping mixing at the blood/Lympholyte-H interface to a minimum. Samples were then centrifuged at 1000g for 20 minutes at room temperature. This results in a clear lymphocyte/monocyte layer, with red cells clumped below the lympholyte-H layer (Figure 2.12).

The lymphocyte/monocyte layer was then carefully removed with a 2ml pasteur pipette and transferred to a clean tube. The cell suspension was then topped up with 5-10ml PBS pH7.4 and centrifuged at 300g for 10 minutes to clear platelet contamination. The supernatant (containing the platelets) was then discarded and the sample was again re-suspended in 5-10ml PBS pH7.4 and centrifuged at 200g for 5 minutes. The resulting supernatant should be clearer at this stage as platelets are removed with each wash. Centrifugation at 200g for 5 minutes was repeated 2-3 times until the supernatant was clear and free of contamination. Cell preparations were then suspended in culture medium prior to use.

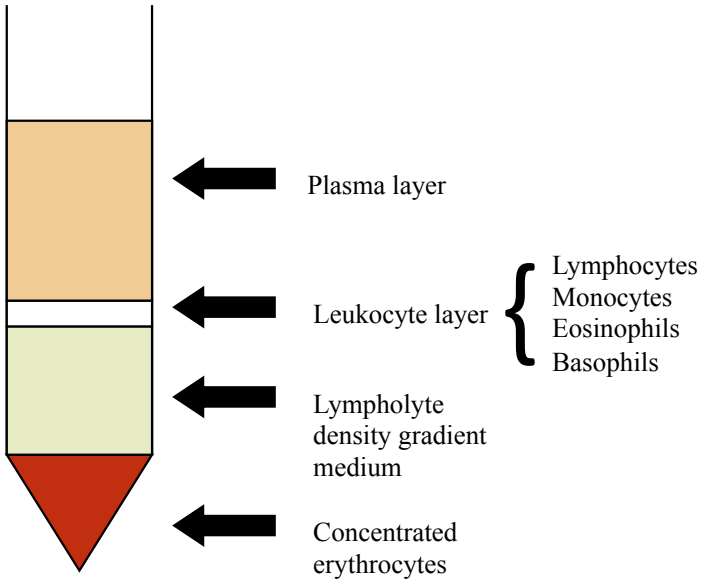


Figure 2.12: Density gradient centrifugation for leukocyte isolation. Following centrifugation at 1000g for 20 minutes the leukocytes form a distinct layer above the density gradient medium that can then be removed for further processing.

2.6.2 T And B Lymphocyte Separation

In the case of cytotoxic crossmatching it is often necessary to further delineate enriched lymphocyte populations into its separate T and B lymphocyte subsets due to the differential expression of MHC class I and II (T lymphocytes express only class I, whereas B lymphocytes express class I and II). Partition of lymphocyte preparations into T and B lymphocyte subsets was achieved using HLA Class I/II dynabeads (Invitrogen, CA, USA). Dynabeads are magnetic particles coated with monoclonal antibody; T-cell specific dynabeads are coated with an anti-CD8 monoclonal antibody and B-cell specific dynabeads are coated with a monoclonal antibody specific for a conserved region of the DRB1 chain of the class II HLA molecule [143].

Purified lymphocyte preparations were re-suspended in 1ml PBS pH 7.4 containing 0.6% citrate and chilled at 4°C for 5 minutes. Next, 15µl either HLA-class I or class II dynabeads were added and the sample was incubated for 5 minutes at 4°C. The sample was placed on a tube roller at this point to ensure complete mixing of cells and dynabeads. Samples were then topped up with 4ml cold PBS pH7.4 containing 0.6% citrate and tubes were placed into a magnetic particle concentrator (MPC) for two minutes. The supernatant was then decanted into a fresh tube and labelled as a B/T-cell depleted fraction, ensuring that the sample tube remains in place inside the MPC at this point or isolated beads and cells will be lost. The original sample tube was then removed from the MPC and the beads were re-suspended in 4ml chilled PBS pH7.4 and returned to the MPC for 1 minute. The supernatant was then discarded, again ensuring that the tube remains inside the MPC. The bead/cell suspension was then suspended in RPMI 1640 containing 2% foetal calf serum (FCS).

2.6.3 Complement Dependant Cytotoxic (CDC) Crossmatch

Complement dependant cytotoxicity (CDC) is an assay used to determine immunological compatibility between potential organ donor and recipient prior to transplant [25]. Binding of immunoglobulin to HLA molecules on the donor cell surface leads to effective cross-linking of IgG by C1q resulting in activation of the classical pathway of the complement cascade and subsequent cell death.

Preparations of separated T and B lymphocytes (see section 2.6.2) were standardised to $2x10^6$/ml in RPMI using a Neubauer counting chamber. CDC plates were first prepared by adding 20µl of mineral oil to each well. The plate was then

prepared with 2µl sample sera, positive and negative control sera into the appropriate wells.

One µl dithiothreitol (DTT) was added to specific wells to differentiate between CDC positivity caused by IgM and IgG. DTT disrupts the inter and intra-chain disulphide bonds prevalent in immunoglobulin structure (refer back to figure 1.13). The concentration of DTT was optimised to 20µM to disrupt the disulphide bond structure of pentameric IgM leaving IgG intact. The plate was then incubated at 37°C for 30 minutes. To each well, 1µl of standardised cells (B or T cells as required) were added and the plates were incubated at 22°C for 60 minutes. Lyophilised rabbit serum (Cedarlane, UK) was reconstituted using 2ml distilled water and 5µl was added to each well as a source of complement. The plates were once again incubated at 22°C for 60 minutes. Fluoroquench (One Lambda Inc, CA, USA), a cocktail of fluorescent dyes to stain lymphocytes and quench background fluorescence, was added to each well (5µl). Cells were then visualised using ultra-violet microscopy. Live cells stain green and dead cells stain red, positivity is determined by calculating the ratio of live/dead cells (figure 2.13).

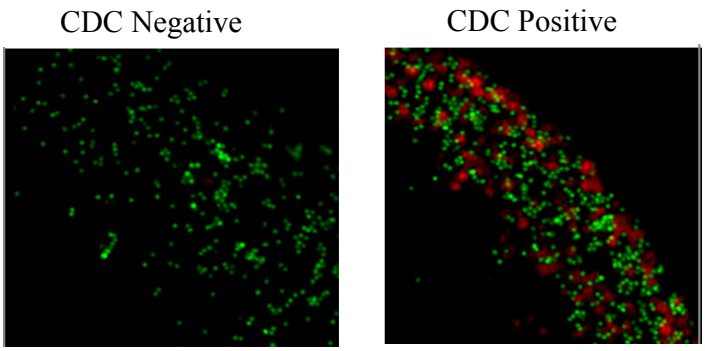


Figure 2.13: Electron microscope images depicting examples of negative and positive cytotoxic crossmatch positive sera. Dead cells stain red under UV light due to ethidium bromide staining of nuclear DNA in ruptured cells.

2.6.4 Flow Cytometry (FC) Crossmatch

Flow cytometry crossmatching (FCXM) provides additional sensitivity compared to CDC. The method relies upon direct detection of donor specific IgG binding to lymphocyte subsets, and is therefore able to detect all bound IgG not just those that are capable of complement fixation [113].

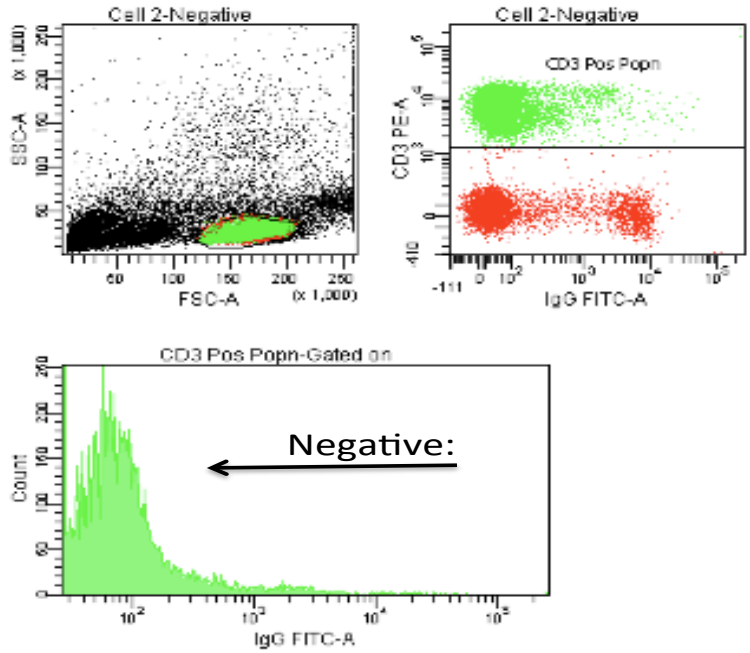
Purified lymphocyte preparations from the potential donor were standardised to $12x10^6$/ml in PBS pH7.4 containing 2% BSA. A negative control serum consisting of a pool of five blood group AB male blood donors previously shown to be negative for anti-HLA reactivity was prepared, as well as a positive control serum which was a pool of ten highly sensitised renal transplant waiting list patients.

Sample tubes were prepared by adding 25µl cells to 25µl serum. Control and test samples were set up in duplicate and then incubated at 22°C for thirty minutes. Samples were then washed three times in PBS pH7.4. To each tube 100µl fluorescein isothiocyanate (FITC) conjugated goat anti-human IgG (working dilution 1:100) was added. Tubes were mixed thoroughly and incubated at 4°C for 15 minutes. Samples were then washed once more in 4ml PBS pH7.4. Then for B cell FCXM 100µl phycoerythrin (PE) conjugated mouse anti-human CD19 (working dilution 1:20) was added. For T cell FCXM 100µl phycoerythrin (PE) conjugated mouse anti-human CD3 (working dilution 1:20) was used. Samples were incubated once more at 4°C for 15 minutes then washed again in 4ml PBS pH7.4. Samples were re-suspended in 200µl PBS pH7.4 containing 2% BSA and 0.01%NaN_3 ready for analysis.

Tests and controls were then analysed on a BD FACSCanto II flow cytometer (Becton Dickinson, UK). For each sample 10,000 individual cells, or events, were counted and the median fluorescence values of controls and test samples were

determined. Figure 2.14 shows examples of negative and positive control samples when analysed using the T-cell specific (CD3) FCXM.

The definition of positivity regarding the flow cytometry crossmatch varies between laboratories and is often dependant upon local policy based upon clinical outcomes. In the Birmingham Histocompatibility and Immunogenetics laboratory crossmatch positivity is calculated as the median fluorescence of the IgG FITC detector divided by the median fluorescence of the negative control sample to provide a ratio termed the relative median fluorescence (RMF). In our laboratory RMF>2.3 denotes a positive crossmatch.



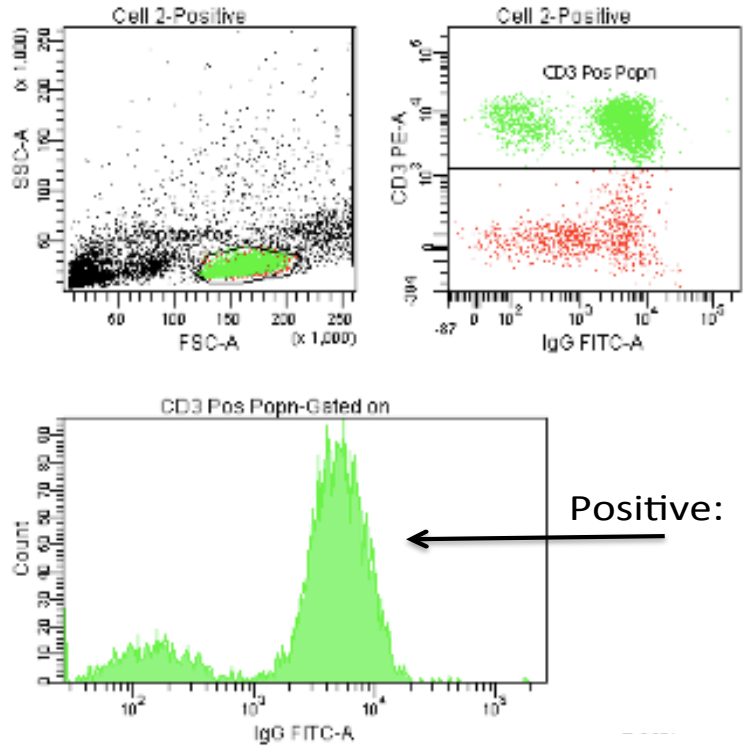


Figure 2.14: Flow cytometry crossmatch histograms. Negative crossmatch (top panel), and positive crossmatch (bottom panel) identified by channel shift for the IgG FITC.

2.7 LUMINEX MICROBEAD ANTIBODY SCREENING AND IDENTIFICATION

As outlined in the introduction, the development of recombinant HLA proteins coupled to microbeads has revolutionised the way in which laboratories are able to define HLA-specific antibodies [114]. At present these microbead preparations are supplied by two manufacturers, One Lambda Inc, (CA, USA), and Gen-Probe, (CA, USA). Although the principle underpinning both manufacturers' assays is the same there are a number of subtle differences in the way in which each assay is prepared and tested. The detailed protocols for each assay is described in this section. It is also important to note that both of these assays were developed purely as a qualitative tool only, the use of these assays to monitor the changes in antibody titre has been outside the scope of the original assay design.

2.7.1 Sample Preparation

Serum samples were taken using BD Vacutainer (Becton Dickinson, UK) 5ml blood collection tubes containing silicone and micronized silica particles which act as a clot activator. All samples were given with full informed and written consent from patients participating in the HLA incompatible transplant programme at University Hospital Coventry and Warwickshire (UHCW), and ethical approval for these studies was applied for and obtained (Coventry Research Ethics Committee- CREC 055/01/03). Serum fractions were taken and frozen in 100µl aliquots until required.

2.7.2 Antibody Screening and Identification- One Lambda Assays

Prior to testing samples were centrifuged at 13,000rpm for ten minutes. The required number of wells was prepared in a 96 well filter microplate (Millipore, UK) by the addition of 200µl One Lambda wash buffer. The wells were then applied to a vacuum to draw the wash buffer through the wells. Finally 20µl buffer was dispensed into each well.

One tube of each required microbead preparation tubes (One Lambda Inc, CA, US) were placed into an ultra-sonic bath and subjected to ultrasonic agitation for five minutes in order to disperse any aggregated beads. The sonicated bead tubes were briefly centrifuged at 13,000rpm for ten seconds then vortex mixed for thirty seconds. To each well, 2µl of microbead preparation was added followed by 8µl of serum. One well containing HLA-specific antibody negative control serum, and one well containing multi-specific HLA antibody positive control serum was set up with each batch of testing. Test plates were then incubated between 18 and 22ºC in the dark for thirty minutes. Following incubation the wells were washed by adding 200µl wash buffer to each well. The plate was then applied to a vacuum to draw through the wash buffer and remove any non-specific binding. The wash step was repeated a further three times to give a total of four washes.

A 1mg/ml stock concentration of phycoerythrin (PE) conjugated goat anti-human IgG is supplied with each One Lambda kit. This reagent is light sensitive and must be stored and used with minimal exposure to natural light. This antibody was then diluted 1in100 with wash buffer to obtain the correct working concentration of 10µg/ml. To each well 40µl of diluted antibody was added and the plates were once again incubated between 18 and 22ºC in the dark for thirty minutes. Following the second

incubation the plates were washed four times as before using the vacuum filtration method, reactions were then re-suspended in 85µl wash buffer. Plates were then analysed using the Luminex™ Xmap 100 platform (Luminex Corp, US) and antibody data was analysed using the HLA Fusion software (One Lambda Inc, US).

The assay set-up was outlined in figure 1.20. Figure 2.15 shows an example of the luminex antibody profile data.

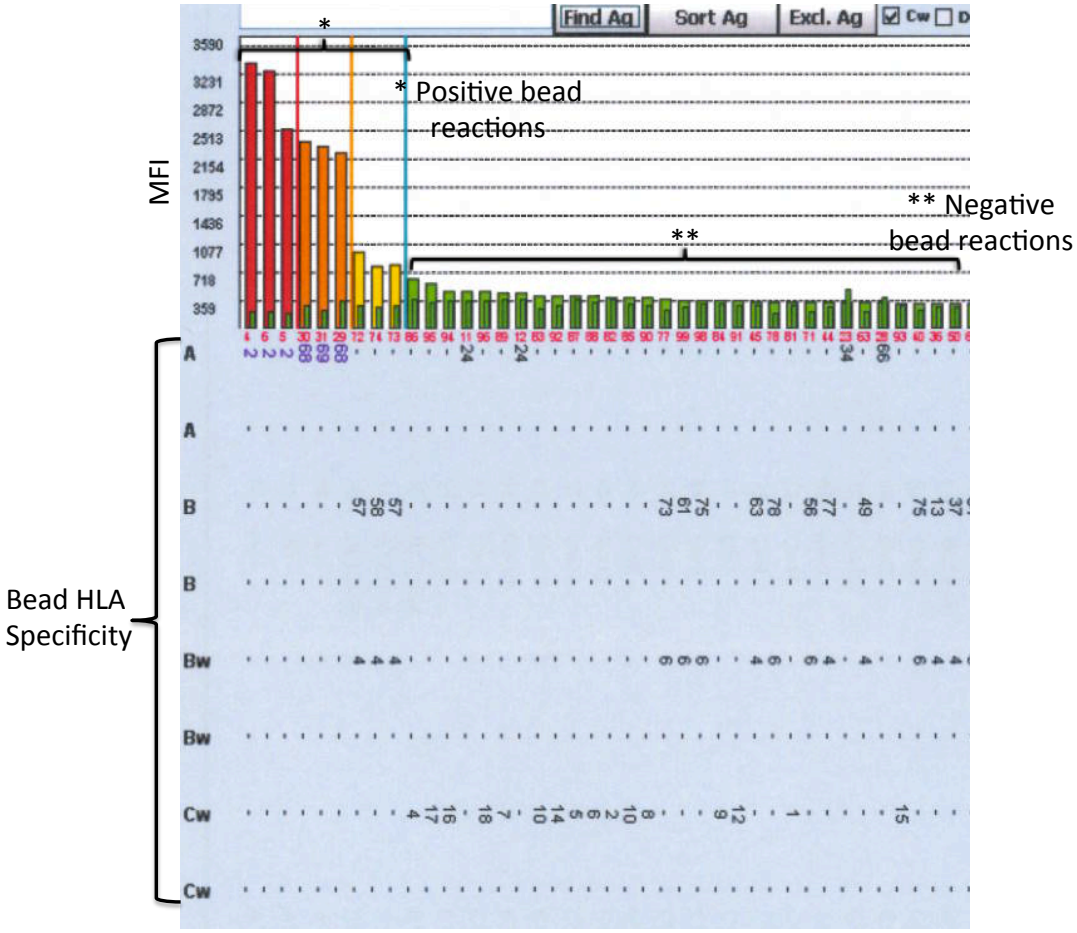


Figure 2.15: Example data from One Lambda single antigen microbead analysis. Class I HLA specific antibody positive profile, the patient reacts positively with beads specific for HLA-A2, A68, A69, B57, and B58. Positivity is determined by increased detection of fluorescently labelled human IgG specific secondary antibody, measured on the y axis as MFI (Mean fluorescence intensity). The principle of the assay is described in detail in section 1.10 and figure 1.18.

2.7.3 Antibody Identification- Gen-Probe Assays

Prior to testing samples and plates were prepared as for the One Lambda assay protocol. Gen-Probe bead preparations were also sonicated, centrifuged, and vortex mixed as for One Lambda bead sets.

To each microplate well, 20µl of microbeads were added, followed by 5µl of patient serum, negative and positive controls as required. Test plates were then incubated between 18 and 22ºC in the dark for thirty minutes. Following incubation, 100µl of wash buffer was added to each well and then drawn through by application of a vacuum. Two further washes were then performed by adding 250µl wash buffer to each well followed by vacuum filtration.

Phycoerythrin conjugated anti-human IgG as supplied by the manufacturer was diluted 1 in 10 with wash buffer, then 20µl is added to each test well. Samples were then incubated for a second time between 18 and 22ºC in the dark for thirty minutes. Following second incubation samples were suspended in a further 100µl wash buffer and analysed on the Luminex™ Xmap 100 platform (Luminex Corp, US). Data were analysed using LifeMatch Quicktype analysis software version 2.2.

2.7.4 IgG Subclass Specific Luminex Microbead Assay

Immunoglobulin G (IgG) can be present in any of four distinct isotypes (IgG1-4, see table 1.5), the presence of each of which confers a distinct set of functional characteristics upon a patients' antibody repertoire. To investigate this we modified the standard microbead analysis method outlined in section 2.7 as follows.

The subclass specific luminex bead assay was carried out using exactly the same protocol as that given above for the use of One Lambda assays. Phycoerythrin (PE) conjugated goat anti-human IgG is substituted with each subclass specific phycoerythrin (PE) conjugated mouse monoclonal antibody (IgG1, clone 4E3; IgG2, clone 31-7-4; IgG3, clone HP6050; IgG4, HP6025, Southern Biotech, Birmingham, AL, USA). The final concentration of each subclass specific antibody was 0.25μg/μl. Samples were analysed as before using the Luminex™ Xmap 100 platform (Luminex Corp, US) and antibody data was analysed using the HLA Fusion software (One Lambda Inc, US).

This modified subclass detection assay was validated by preparing a panel of microbeads with each one coated with a separate IgG subclass anibody derived from the serum of multiple myeloma patients. The full details of this validation exercise are given in chapters 7 and 8. The method by which these proteins were coupled to microbeads is given below.

2.7.5 Coupling Protein to Luminex Microspheres

To validate the modified assay to detect IgG subclass binding to microbeads a validation set of beads was prepared. Fresh microbead populations were activated and then coupled with IgG proteins of known IgG subclass. These had been isolated from the serum of patients with multiple myeloma. These antibody samples were kindly provided by Dr Mark Cobbold and Dr Margaret Goodall from the University of Birmingham, UK.

All buffers used in the process of coupling protein to luminex microspheres are listed in Table 2.4.

Table 2.4: Buffers used for the process of coupling proteins to microbeads.

Buffer	Composition
Luminex Buffer	1L PBS 2g BSA (Sigma A4503) 500µl Tween20 (Sigma P.1379) 5ml 10% NaN_3 Filter through 0.2µm and store at 2-8°C
Activation Buffer	3g NaH_2PO_4 (Sigma S0751) 250ml distilled water adjust pH to 6.2 with 1M NaOH
Wash and Storage Buffer	200ml PBS 2g BSA (Sigma A4503) 100µl Tween20 (Sigma P.1379) 2ml 10% NaN_3

PBS, activation, wash, and storage buffers were prepared as listed in table 2.4. The region numbers of the microspheres were chosen such that there would be no coincidence with other microsphere regions, this is particularly important when designing multiplex analysis. Each isolated protein was coated onto its own unique microsphere region. The luminex analyser is able to sort individual beads within a set by virtue of unique fluorochrome ratios with which the beads are impregnated. Each ratio corresponds to an individual detection region, which the luminex analyser is able to discriminate.

Each region specific container of microspheres were vortex mixed for two minutes then centrifuged at 13,000 rpm followed by sonication for five minutes in order to fully disperse the microsphere pellet. Two hundred microlitres of each microsphere (approximately 2.6 $x10^6$ microspheres/ ml) were dispensed into separate wells of a 96 well filter plate (Millipore, MSVHHTS00), this provided enough beads for around 50 tests. The fluid was aspirated from each well using a Millipore vacuum manifold leaving the microspheres on the plate filter mesh. The beads were then washed once by

adding 200µl activation buffer to each well followed by vacuum filtration. Wells were then re-suspended in 200µl activation buffer.

Bead activation cocktail was then prepared by first dissolving 5mg N-hydroxysulfosuccinimide sodium salt (Sulpho-NHS, Sigma, UK #56485) in 100µl activation buffer to give a 50mg/ml solution. Similarly, 5mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hyrdrochloride (EDC, Sigma, UK #6383) was dissolved in 100µl activation buffer to give a 50mg/ml solution. Final coupling buffer was prepared by adding 10µl sulpho-NHS and 10µl EDC to 80µl activation buffer per microsphere (scaled up for coupling more than one microsphere region).

The activation buffer was aspirated from the microplate using the vacuum manifold. To each well of microspheres 100µl coupling buffer was added. The plate was then agitated gently in the dark at room temperature for 30 minutes. Excess coupling buffer was then removed by vacuum filtration.

Proteins to be coupled, in this case individual IgG preparations derived from multiple myeloma patients, were prepared by diluting in PBS to a concentration of 1mg/ml and 100µl of each protein suspension was added to the appropriate microsphere well. The plate was then incubated in the dark for 3 hours at room temperature and under constant agitation. Wells were then washed three times using 200µl storage buffer and vacuum filtration. Microspheres were then re-suspended in 200µl storage buffer by vigorous vortex mixing and transferred to sterile 2ml microtubes. This process was repeated twice more to ensure no microspheres were left in the wells. There was then 600µl of microspheres at a concentration of $4x10^6$ microspheres/ ml ready for use. Beads can be stored at 2-6°C for up to 6 months.

2.7.6 C1q Screen Assay

The ability of antibody to fix complement via the classical pathway is dependent upon the isotype of the antibody. As discussed earlier IgG1 and IgG3 are potent activators of complement whereas IgG2 and IgG4 are less so. To distinguish between complement and non-complement fixing anti-HLA antibodies One Lambda developed the C1qScreen™ assay, which uses microbeads to identify the specificities of anti-HLA antibodies capable of crosslinking with C1q and initiating the classical complement activation pathway (figure 2.16).

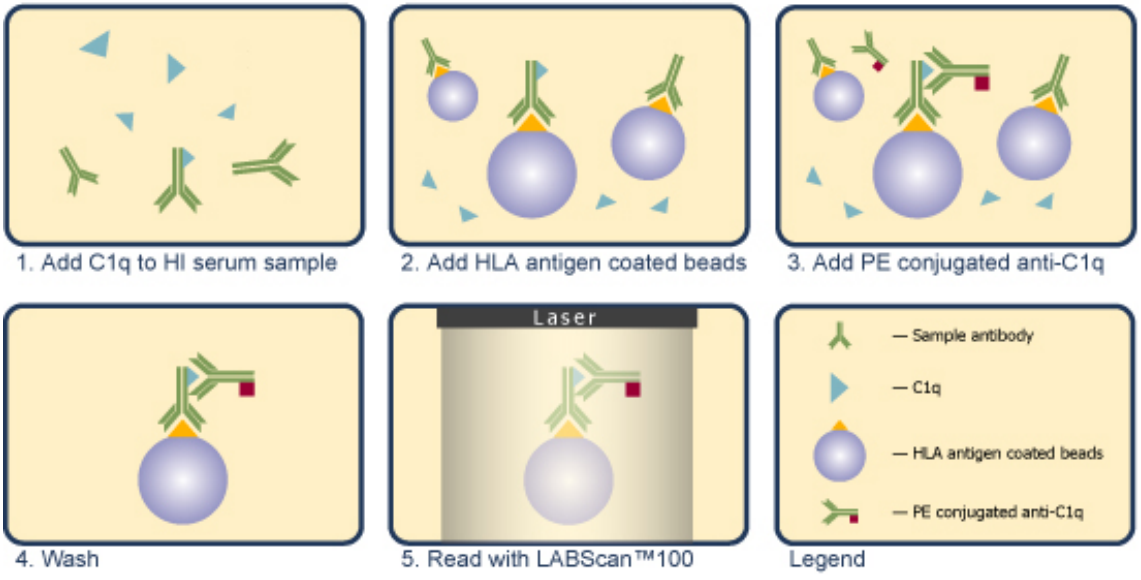


Figure 2.16: Principle of the One Lambda C1qScreen™ assay. The test allows the direct identification of those anti-HLA antibody specificities which have the capacity to bind human C1q and thus initiate the classical pathway of complement activation.

One Lambda C1qScreen™ assay was used to determine the presence of complement binding HLA specific antibodies in human serum [122-124]. To prepare the serum for analysis 40µl serum was transferred to a fresh 1.5ml Eppendorf tube and incubated in a water bath at 56ºC for thirty minutes in order to inactivate endogenous complement present in sera. The sample was then centrifuged at 13,000rpm for 10 minutes and the clear serum supernatant (approximately 30µl) was decanted into a fresh 1.5ml Eppendorf tube and then kept chilled on ice.

Human C1q protein was reconstituted in 10mM HEPES pH7.2 at a volume of 1µl per test sample. The reconstituted C1q was then diluted a further four fold in 10mM HEPES pH7.2, thoroughly mixed via vortex and kept on ice prior to use.

Next, 5µl of the diluted human C1q was added to designated separate wells of a 96 well microtitre filter plate, and 5µl of heat inactivated serum sample was added to each appropriate well. The plate was gently mixed before 5µl of class I or class II single antigen coated beads (LS1A04 / LS2A01, One Lambda Inc) was added to each well. The plate was then gently mixed by vortex mixing and incubated at room temperature for 20 minutes. The plate must be constantly agitated and protected from light during the incubation period.

Following incubation, 5µl of PE-conjugated anti-C1q as supplied by the manufacturer was added to each test well and thoroughly vortex mixed. Again the plate was incubated in the dark at room temperature for 20 minutes under constant agitation. To each well, 80µl of PBS pH7.4 was added and the supernatant was removed by vacuum filtration. A further 80µl of PBS pH7.4 was added to each well and the plate was then analysed on the Luminex™ Xmap 100 platform (Luminex Corp, US), and antibody data was analysed using the HLA Fusion software (One Lambda Inc, CA, US). A positive reaction threshold of >500 MFI was applied to all assays.

2.8 PROTOCOLS TO MANUFACTURE COLUMNS WITH BOUND sHLA

Current methods to remove HLA-specific antibody from patient blood both prior to and following solid organ transplantation are both inefficient and non-selective. Therefore humoral immunity of the patient is often compromised leading to increased risk of secondary complication such as infection. In order to remove anti-HLA antibody in a highly effective and specific manner we sought to design antibody removal columns using sepharose as a carrier matrix and soluble HLA proteins as the immobilised target antigen over which patient plasma would be passed. We have abbreviated the name of this column to soluble HLA antibody removal column (SHARC). Figure 2.17 shows both the mini- and clinical scale SHARC columns, the production and use of each of these is detailed in the following section.

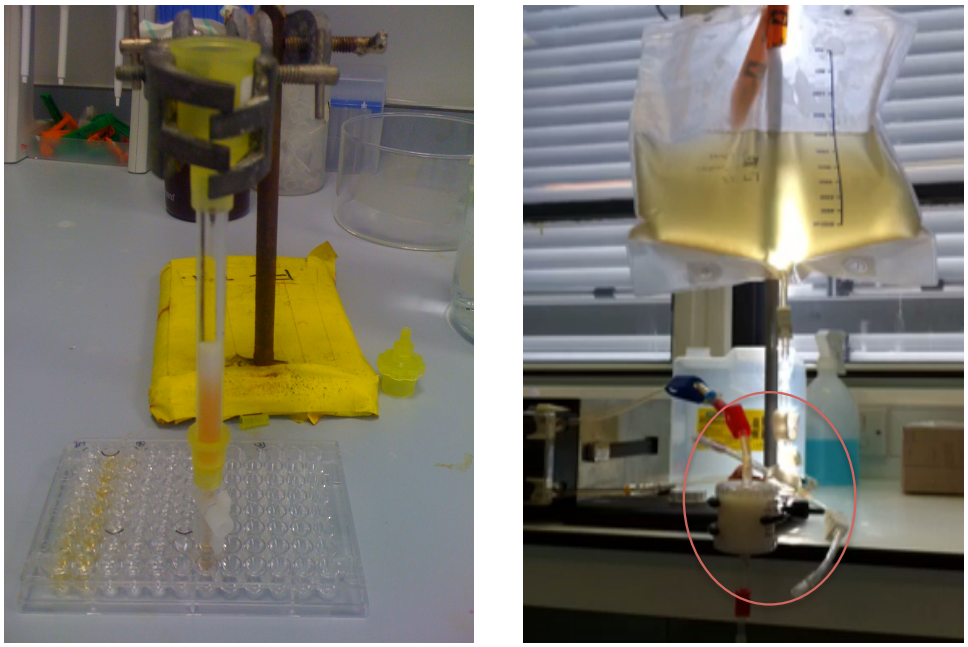


Figure 2.17: Examples of SHARC columns used in this project. The left panel show a 1ml column used to deplete small volume patient samples of HLA-specific antibody. The right panel shows a 50ml clinical scale SHARC column (circled) used to deplete larger volume patient samples.

2.8.1 Mini HLA-column Coupling Protocol

The term mini HLA-column is used to describe sepharose coupled with HLA protein which is then packed into 2ml chromatography columns for the purpose of selectively depleting small volumes of patient serum or plasma samples of HLA-specific antibody. Typically mini-HLA columns contain between 100µg and 1mg of sHLA protein coupled to up to 1ml of activated sepharose.

To hydrate the sepharose, 200mg cyanogen-bromide (CNBR) activated sepharose (Sigma-Aldrich, UK) was resuspended in 2ml of 1mM hydrogen chloride (HCl) and chilled on ice for 30 minutes. The sepharose was centrifuged at 3000rpm for 10 minutes then the supernatant was decanted. The sepharose was resuspended in suspension buffer consisting of 50mM HEPES pH7.8, and 100mM NaCl (Sigma-Aldrich, UK) then centrifuged again at 3000rpm for 10 minutes. This stage was repeated twice more for a total of three washes. Next, 500µl suspension buffer was added to a clean 1.5ml Eppendorf tube and the CNBR resin was transferred into this to give an approximate total volume of 1ml and an approximate sepharose concentration of 2mg/ml.

Soluble HLA (sHLA) was added to the sepharose at a concentration of 2mg per 1ml of sepharose (for example add 200µg of sHLA to 100µl of activated sepharose). The sHLA/sepharose mix was incubated at 4^{o}C for two hours whilst rotating constantly. The sepharose was centrifuged gently at 500rpm for 5 minutes then the supernatant was decanted into a fresh eppendorf tube. The A_{280} reading of the supernatant by nanodrop analysis (see section 2.5.4) was used to determine the amount of sHLA bound to the sepharose, aiming for >80% coupling efficiency. If <80% coupling was observed, the sample was incubated again for two hours at 4^{o}C, then centrifuged at 500rpm for 5

minutes then protein concentration in the supernatant was determined. If required the steps were repeated again until >80% coupling efficiency was achieved.

To deactivate the sepharose 1ml of 1M ethanolamine was added to the sepharose pellet and incubated at 4^{o}C overnight whilst constantly rotating. The sample was then centrifuged at 500rpm for 5 minutes and the supernatant was then carefully discarded. The pellet was then resuspended in 1ml PBS pH7.4 and centrifuged at 500rpm for 5 minutes. The supernatant was again discarded and the pellet was rinsed once more in PBS pH7.4 by addition of 1ml followed by centrifugation at 500rpm for 5 minutes. The pellet was then resuspended in an approximate equal volume of PBS pH7.4 and spiked with 0.02% sodium azide (NaN_3) to act as a preservative.

HLA protein coupled sepharose was then packed into a 2ml affinity chromatography column (Bio-rad Laboratories, CA, USA). To prepare the column filter mesh, 2ml of PBS was dispensed into the column. The flow tap was opened and the PBS was allowed to flow through the column and fully hydrate the filter. Up to 1ml of HLA coupled sepahrose was then added to the column, ensuring that the flow tap was in the closed position. The sepharose was then allowed to settle for up to one hour at room temperature. The flow tap was then opened and the excess PBS from the sepharose was allowed to flow through. The flow tap was closed before the PBS level dropped below the level of the sepharose, under no circumstances should the sepharose be allowed to dry out. A further 2ml of PBS was then added and allowed to flow through to the same level as before. The column was then resuspended in 2ml PBS containing 0.01% NaN_3 and stored at 4^{o}C until ready for use.

2.8.2 Clinical Scale HLA-column Coupling Protocol

We define the term clinical scale HLA-column as being the required volume of HLA protein coupled to sepahrose to effectively deplete 2-3 litres of patient plasma of HLA-specific antibody. The calculations to determine the required protein concentration to achieve this were determined via extrapolation of mini-HLA column data and subsequent empirical testing using larger scale HLA-columns. The details of these validation experiments is provided in chapter 8, but a 60ml sepahorose column containing 50mg sHLA was used for clinical scale depletion experiments.

For clinical scale protein coupling the same protocol was used as for mini-column coupling, with one minor amendment being the addition of sHLA at a reduced concentration of 1mg per 1ml of swollen matrix. All other incubation times and wash steps were kept the same but volumes were scaled up accordingly.

Column packing was also performed as per the mini-column protocol but wash volumes were scaled up to take into account the 50ml volume capacity of the clinical scale column.

2.8.3 Affinity Columns: HLA Specific Antibody Depletion using SHARC

Up to 5ml of patient sera was loaded into the column reservoir and allowed to drip through by gravity by releasing the flow tap at the bottom. Fractions were collected drop wise into 96 well ELISA plates so that microbead analysis could be performed retrospectively (3 drops per well which was the approximate equivalent of 150µl). Flow taps were closed when the sample reached the upper limit of the protein coupled resin

inside the column chamber. The column was then washed with up to 7ml of PBS pH7.4 with fractions taken once more into ELISA plates.

HLA specific antibodies bound to the protein/sepharose matrix were then eluted by introducing an alkaline pH to the column. Acid elution of HLA-specific antibodies is unsuitable as sHLA is denatured at a pH of 6 or below. An elution buffer of 100mM glycine pH11.0 was added, typically in the volume range 8-10ml. Elution fractions were collected as before, with 35µl 1M tris pH7 pre-loaded into each well to assist in neutralising the antibody fractions. Each fraction was retrospectively tested by microbead analysis for presence of purified HLA specific antibodies.

HLA columns were then re-equilibrated by addition and flow through of up to 10ml PBS pH7.4, followed by a long-term storage buffer of 5ml PBS pH7.4 containing 0.01% NaN_3. Columns were then stored at 4^oC.

Column integrity following repeated rounds of antibody depletion and elution was assessed by repeated depletion of a standard concentration of the HLA-class specific monoclonal antibodies W6/32 and anti-β_2M.

2.8.4 Antibody Depletion and Isolation using clinical scale SHARC

For clinical scale HLA columns between 2 and 3 litres of diluted plasma effluent were run through the column at a flow rate of 50ml/min using a Watson Marlow 501H peristaltic blood pump (Watson Marlow, Portsmouth, UK). Pressure monitors were set up both proximally and distally to the HLA column in order to monitor the fluid pressure through the column and potentially identify any build up in pressure indicative of column blockage (figure 2.18).

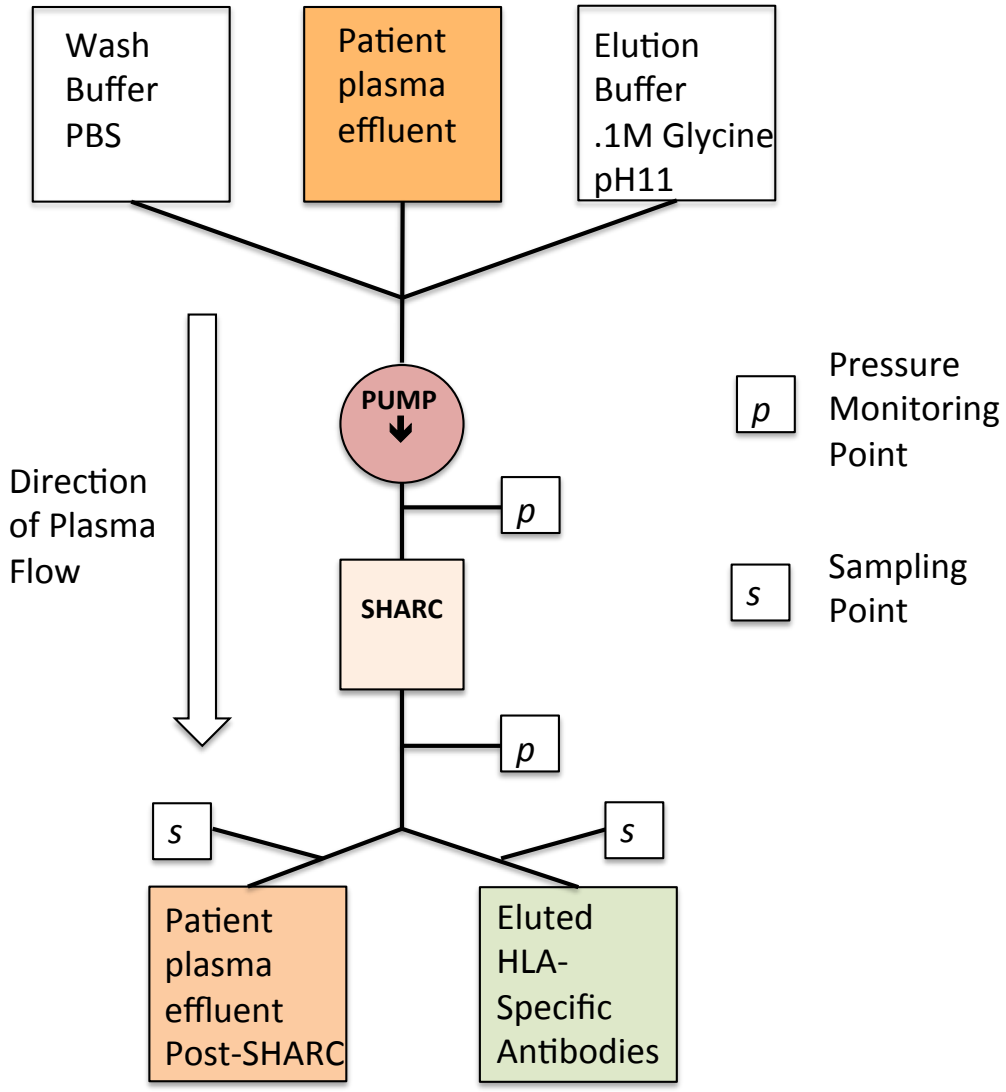


Figure 2.18: Circuit Design for Clinical Scale HLA-Specific Antibody Removal. The circuit was designed to incorporate pressure monitor sensors both proximally and distally to the SHARC. The circuit can be diverted such that post depletion samples and eluted antibody preparations can be collected separately.

Following the run through of patient material the circuit was switched to allow 1-2 litres of PBS pH7.4 to run through and remove any non-specific binding to the HLA column. Antibody elution was achieved by running through 2 litres of 100mM glycine pH11.0.

To neutralize the antibody preparation at this stage 20% w/v 1M Tris pH7 was added. The column was then re-equillibrated using 2 litres PBS pH7.4 , then 2 litres of PBS pH7.4 containing 0.01% NaN_3 for long-term storage.

Chapter 3

SINGLE ANTIGEN LUMINEX BEADS-

PRACTICAL CONSIDERATIONS.

3.1 INTRODUCTION

The central dogma that pre-existing donor-specific (HLA and ABO) antibodies (DSA) are a bar to renal transplantation is steadily being dismantled. It is becoming apparent that *quantity* in addition to *specificity* is a critical factor in determining whether to transplant a patient with HLA-specific antibodies. On this basis pretransplant antibody removal or reduction can eliminate hyperacute rejection. Despite this there remains increased risks of graft damage and acute rejection and these are likely to be dependent on the amount of antibody present at the time of transplantation and post-transplant resynthesis of DSA. For these reasons accurate and reproducible measurement of HLA specific antibody together with a strategy of antibody monitoring is required.

The ability to measure circulating levels of DSA, in both the pre- and post-transplantation phase is critical to understanding the patient's immunological status. The number of removal cycles (e.g plasma exchange cycles) will depend on the amount and rate of re-synthesis of the pre-existing DSA. During antibody removal the effectiveness of the procedure can be determined by specific measurements, ideally before and after each treatment, finally reaching a level which is considered safe and manageable. Post transplant monitoring identifies the status of the patient's response, for example, early immunological signs of rejection, and can be used to guide treatment where appropriate [91]. Complement dependent cytotoxicity (CDC) and later flow cytometry (FC) were first used to monitor HLA-specific antibody levels. In comparison to microbead assays, both lack sensitivity and require viable donor or third party lymphocytes and are cumbersome to perform on a daily basis. These are also difficult to standardise because of variability seen with cells from different individuals [115]. More recently assays based on purified HLA are in widespread use, and of these the

bead-based assays are the most used. Bead assays are a development of FC but use antigen coated beads instead of lymphocytes as targets for HLA-specific antibody detection [114]. These are standardised, quality controlled reagents which have enabled us to monitor AiT on a real-time basis. However, the use of this assay for antibody quantitation rather than detection has not been validated. There are many variables which need to be considered, such as different antigen coating levels between beads (specificities), the linearity of dose response, and the level at which the assay saturates. In this chapter I show how these factors determine antibody measurement so that the bead based assay can be standardised, and monitor changes in DSA levels to inform effective transplant management.

3.2 PATIENTS AND METHODS.

3.2.1 Microbead Assays.

HLA class I and class II specific antibodies were analysed using recombinant single antigen microbead assays manufactured by OneLambda Inc. (Canoga Park, CA) and analysed on the Luminex Xmap 200 platform. Antibody binding was measured as raw fluoresence to avoid differences in background binding seen with different sera which disproportionately influences relative fluorescence, a particular problem associated with plasma exchange [91, 116].

3.2.2 Maximum antibody binding capacity.

The relative density of HLA antigen bound to each bead was determined using monoclonal anti-HLA antibodies (Mab). For class I, PE-conjugated W6/32 monoclonal

antibody (Cedarlane Labs, Ontario, Canada) and PE-conjugated polyclonal Beta-2-microglobulin (β_2M) specific antibodies (AbCam, Cambridge, MA) were used. For class II we used separate PE conjugated anti-HLA-DP, DR, and DQ monoclonal antibodies (AbCam, Cambridge, MA). All monoclonal antibodies were tested against each single antigen bead assay and standard curves were constructed to determine the maximum antibody binding capacity (ABC) for each bead (dilutions used were 1, 0.5, 0.25, 0.125, 0.031, 0.016mg/ml). The distribution of mean channel fluoescence intensity (MFI) values corresponding to the maximum ABC of all beads was used to normalise subsequent serological measurements to the 75th percentile.

3.2.3 Binding characteristics of human antibodies.

All assays were performed using serum/bead ratios in accordance with the manufacturer's instructions. Raw MFI values were used to track changes in DSA and TPA levels over the course of the transplant as previously described [91, 116].

The binding capacity of each patient's serum was investigated by testing serial dilutions of patient sample (tested at neat then dilution with AB serum at 1:5, 1:10, 1:50, 1:100, 1:250) and construction of separate standard curves for class I and Class II HLA.

3.2.4 Patients.

Patients were selected for HLA antibody incompatible transplantation if they displayed current reactivity with donor specific HLA antigen as measured by CDC crossmatch, flow cytometry crossmatch, or by luminex single antigen microbead assay. Patients were treated prior to transplant with alternate day sessions of double filtration

plasmapheresis (DFPP). The number of sessions administered was dependant upon starting levels of DSA with five being the most frequent. Immunosupression was given as previously described [91, 116].

Post transplant samples were tested for DSA and TPA levels daily for the first two weeks then three times a week for the following two weeks. All antibody analysis was carried out on a real-time basis as the results were used in acute patient management. Diagnosis and treatment of rejection are as previously described [91, 116].

3.3 RESULTS.

3.3.1 Saturation of the binding capacity of the single antigen bead assay.

Maximum antibody binding capacity (ABC) of the OneLambda HLA class I single antigen bead assay was standardised by the use of PE conjugated anti-HLA class I (w6/32) and also anti-beta-2 microglobulin (B_2M). HLA class II ABC was determined using anti HLA-DR, DP and DQ PE conjugated monoclonal antibodies (AbCam, UK). Standard curves were constructed by testing against serial dilutions of each monoclonal antibody in order to standardise ABC across all beads (Figure 3.1).

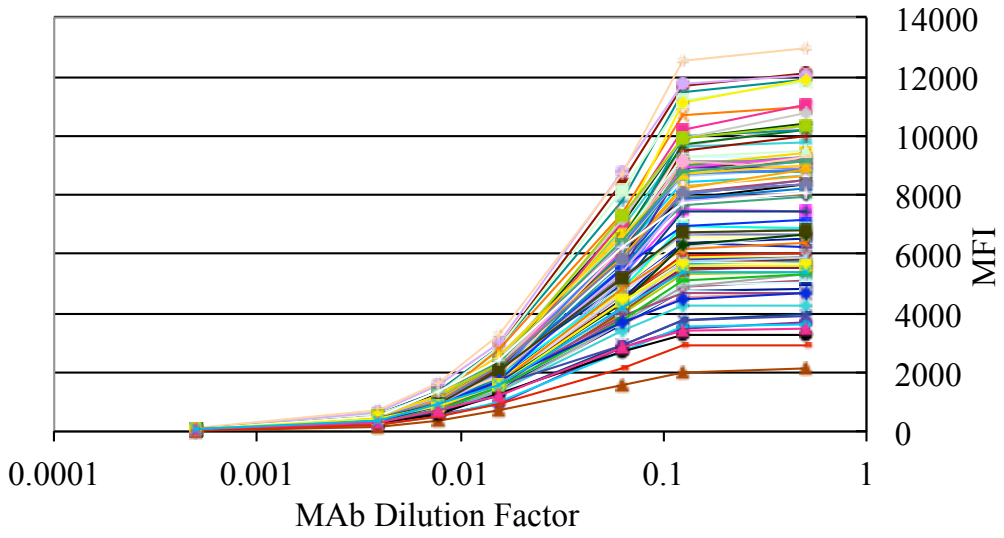


Figure 3.1: Typical standard curve showing the standardisation of HLA class I single antigen beads. This example uses the rabbit polyclonal antibody specific for β_2M. Saturation of available binding sites can be seen by the flattening out of the standard curve at higher concentrations of antibody.

3.3.2 Binding characteristics of human sera.

At higher concentrations of antibody, binding capacity may appear reduced as all available antibody binding sites are occupied and the excess antibody competitively inhibits binding, this observation can be referred to as the 'high dose hook effect'[118].

To demonstrate the high dose hook effect in a clinical situation figure 3.2 shows the standard curve constructed using serial dilutions of a post-transplant sample from patient 039. At neat concentration the DR4 DSA in the patient saturates the available binding sites on the DR4 specific single antigen beads, and in one case this results in a

false low MFI reading. The standard curve indicates that at a dilution factor of 1in10 the DSA is measurable within the linear region of the binding curve and accurate MFI readings can be obtained. The importance of the linear region of the antibody binding curve is discussed in greater detail in chapter 5.

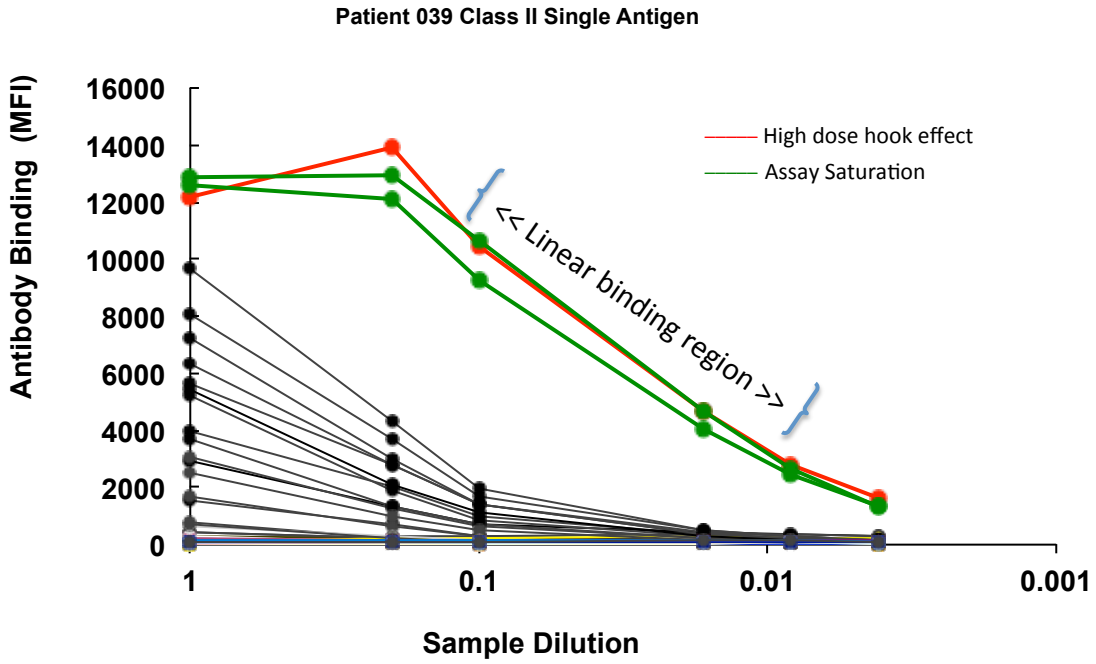


Figure 3.2: Standard curve constructed using serial dilution of a post transplant serum sample. DR4 DSA (colour) displays saturation of binding sites at neat dilution (green)- and in the case of one bead a false low reading: the 'high dose hook effect' (red). The linear binding region, the area of the curve in which MFI increases in correlation with antibody concentration, is also highlighted for the DR4 positive beads.

Failure to recognise the presence of the high dose hook effect may have serious implications on patient management particularly in the post transplant period. Figure 3.3 shows the post transplant antibody profile of case 039. At day 7 this patient had

confirmed rejection with rise in serum creatinine and fall in urine output. Antibody levels appeared to have fallen sharply at onset of rejection. Re-testing of patient sera at a dilution factor of 1in10 revealed a steady rise in DSA levels from day 5 onwards in accordance with deteriorating graft function.

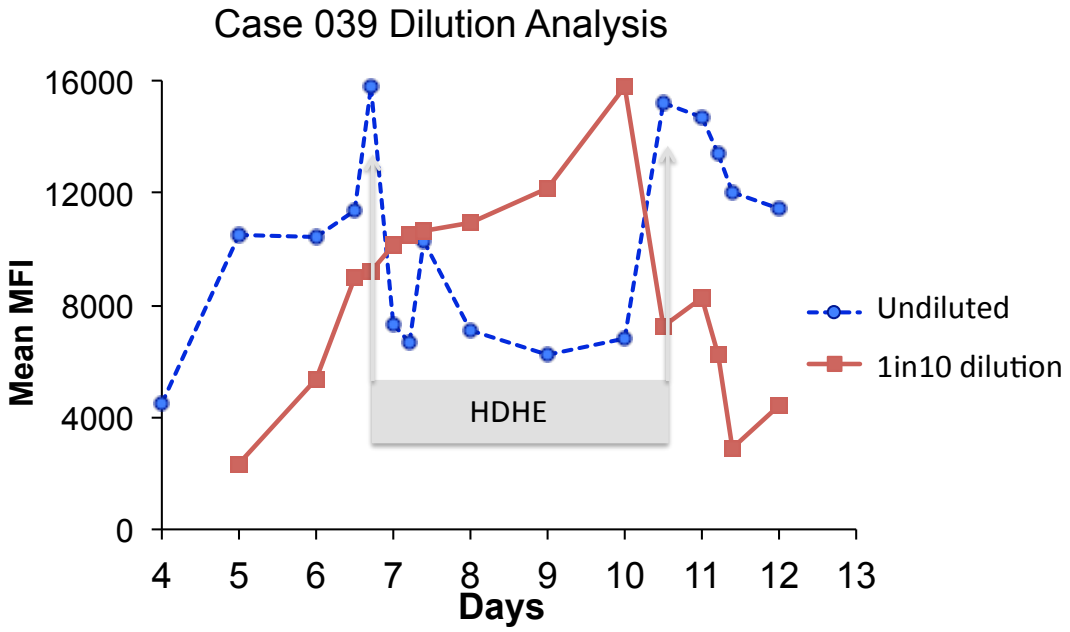


Figure 3.3: The impact of the high dose hook effect on the interpretation of changes in antibody level. Testing without dilution appears to show a sharp fall in antibody levels, however testing at 1 in10 dilution revealed a steady rise in DSA level consistent with deteriorating graft function. The points between which the high dose hook effect affects the antibody levels detected in the microbead assay is highlighted.

The high dose hook effect can be observed most markedly during the initial rounds of pre transplant DFPP. In the case of high titre DSA saturating bead capacity, DFPP results in a false rise in MFI. This can be corrected by dilution of patient serum as shown in figure 3.4.

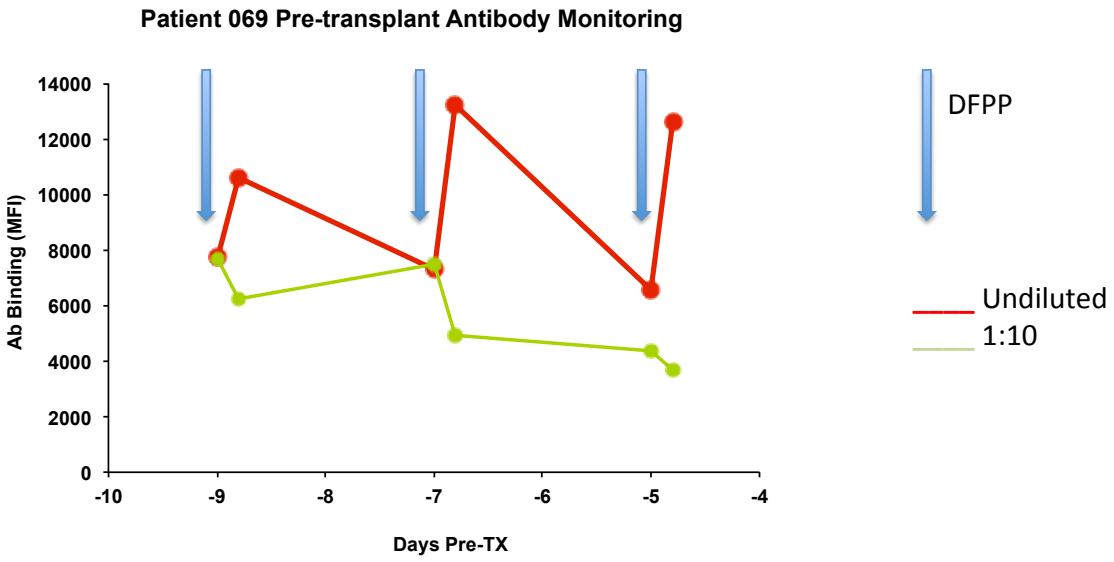


Figure 3.4: The impact of the High Dose Hook Effect in the pre-transplant monitoring period. With each round of DFPP the MFI rises when tested using undiluted serum suggesting ineffective antibody removal. Testing at 1 in10 serum dilution reveals the presence of the HDHE masking the true fall in antibody level.

3.3.3 Effect of correcting for variation in antigen density.

Local studies performed which identify the variation in antigen expression on single antigen beads have shown there can be a large variation in expression level between beads (figure 3.1). By saturating the available binding sites with the use of monoclonal antibodies we have shown that with the One Lambda single antigen assay there is over a twelve-fold difference in expression level between the highest and lowest

density beads. The variation in antigen density that is inherent in the single antigen assay has a profound effect upon maximum ABC.

This knowledge is vital when monitoring accurately the changes in MFI readings in transplant recipients. Figure 3.5 shows the effect of correcting for antigen bead density for patient 035 involved in our HLA antibody incompatible transplant scheme. Although in this case both corrected and uncorrected antibody profiles follow the same general pattern, correction for antigen density reveals that both DSA and TPA were of very similar level. DSA levels appear to be higher than TPA levels until application of correction factors reveals the true titre profile.

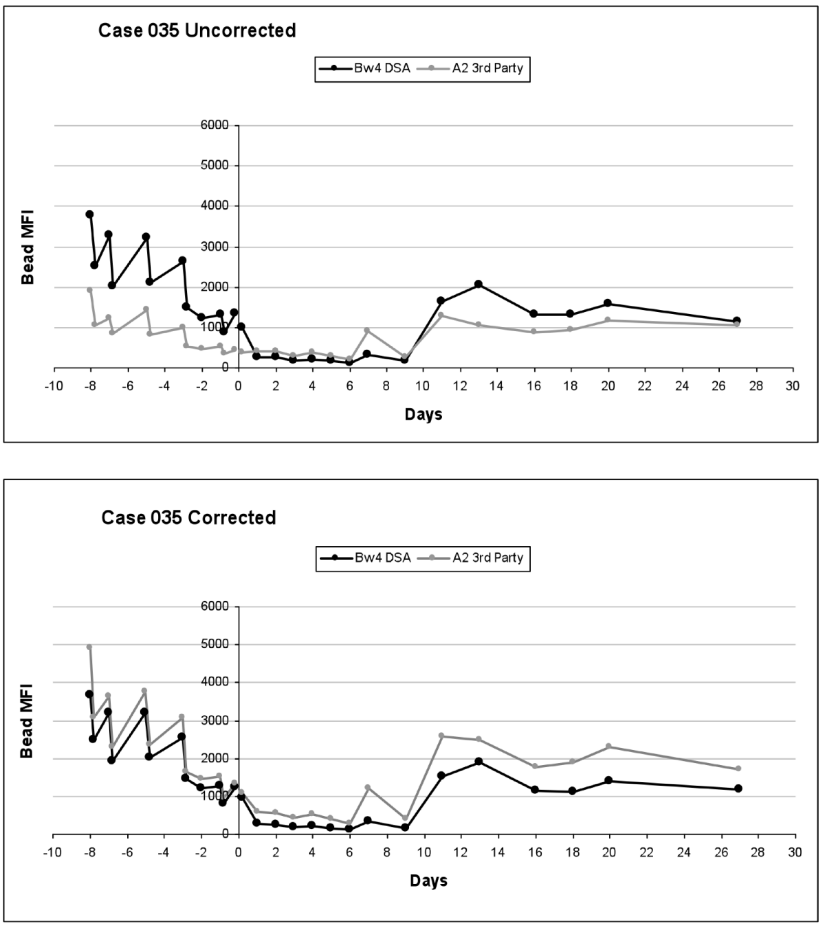


Figure 3.5: Effect of correcting for antigen density. When corrected MFIs are used (bottom panel) we can observe that 3rd party specificities were not of higher level than DSA pre-transplant.

3.3.4 Use of saturation ratios to reveal confounded antibody specificities.

The use of monoclonal antibodies to obtain bead saturation MFI values as an indicator of antigen density also provides a means by which antibody specificities which are confounded by the presence of other specificities can be positively identified. This is particularly apparent when dealing with DSA and TPA directed against the HLA-DP protein. The polymorphism displayed by HLA-DP is unique amongst HLA loci, with almost all known polymorphism attributable to combinations of several different amino acid motifs at six sequence positions [144, 145]. These common epitopes are outlined in table 3.1. As a consequence of this, single antigen microbeads are not always able to conclusively define DSA in the presence of other TPA directed against HLA-DP locus specificities.

Table 3.1: HLA-DP Polymorphic epitopes. Polymorphism is limited to six key amino acid positions.

	Epitope amino acid position					
DPB1*	.8-11	33-36	55-57	65-69	76	84-87
01:01	VY-G	E-YA	AAE	I--K	V	DEAV
02:01	LF-G	E-FV	DEE	I--E	M	GGPM
02:02	LF-G	E-LV	DEE	I--E	M	GGPM
03:01	VY-L	E-FV	DED	L--K	V	DEAV
04:01	LF-G	E-FA	AAE	I--K	M	GGPM
04:02	LF-G	E-FV	DEE	I--K	M	GGPM
05:01	LF-G	E-LV	EAE	I--K	M	DEAV
06:01	VY-L	E-FV	DED	L--E	M	DEAV
08:01	LF-G	E-FV	DEE	I--E	V	DEAV
09:01	VH-L	E-FV	DED	I--E	V	DEAV
10:01	VH-L	E-FV	DEE	I--E	V	DEAV
11:01	VY-L	Q-YA	AAE	L--R	M	DEAV
13:01	VY-L	E-YA	AAE	I--E	I	DEAV
14:01	VH-L	E-FV	DED	L--K	V	DEAV
15:01	VY-G	Q-YA	AAE	L--R	M	VGPM
16:01	LF-G	E-FV	DEE	I--E	M	DEAV
17:01	VH-L	E-FV	DED	I--E	M	DEAV
18:01	VY-G	E-FV	DEE	I--K	M	VGPM
19:01	LF-G	E-FV	EAE	I--E	I	DEAV
20:01	VY-L	E-FV	DED	L--K	M	DEAV
21:01	VY-L	E-LV	EAE	I--K	M	DEAV
22:01	LF-G	E-LV	EAE	I--E	M	DEAV
23:01	LF-G	E-FV	AAE	I--K	M	GGPM

In our transplant program so far there are 5 patients whose sole DSA is directed against HLA-DP. The example shown in figure 3.6 highlights the antibody profile of a renal patient who appears to have raised antibodies via a previous mismatched renal allograft to the 84-87 DEAV motif. This motif is present in DP1,3,5,6,8,9,10,11,13,14,16,17,19,20,21, and 22 antigens, and the patient reacts against all of these specificities (figure 3.6A). Presence of antibody specific for this motif will potentially mask antibodies specific for many other immunogenic motifs, for example both 8-11VY-L and 55-57DED would be confounded. By dividing the sample MFI by the previously determined saturation MFI for each bead a saturation ratio is obtained which can be used to accurately define reactivity between patients (figure 3.6B,C). If the antibody level is high it may be necessary to dilute the patient sample to negate the high dose hook effect. Application of these factors provides clear 'groups' of saturation ratios which correlate to exact antibody specificities (figure 3.6D).

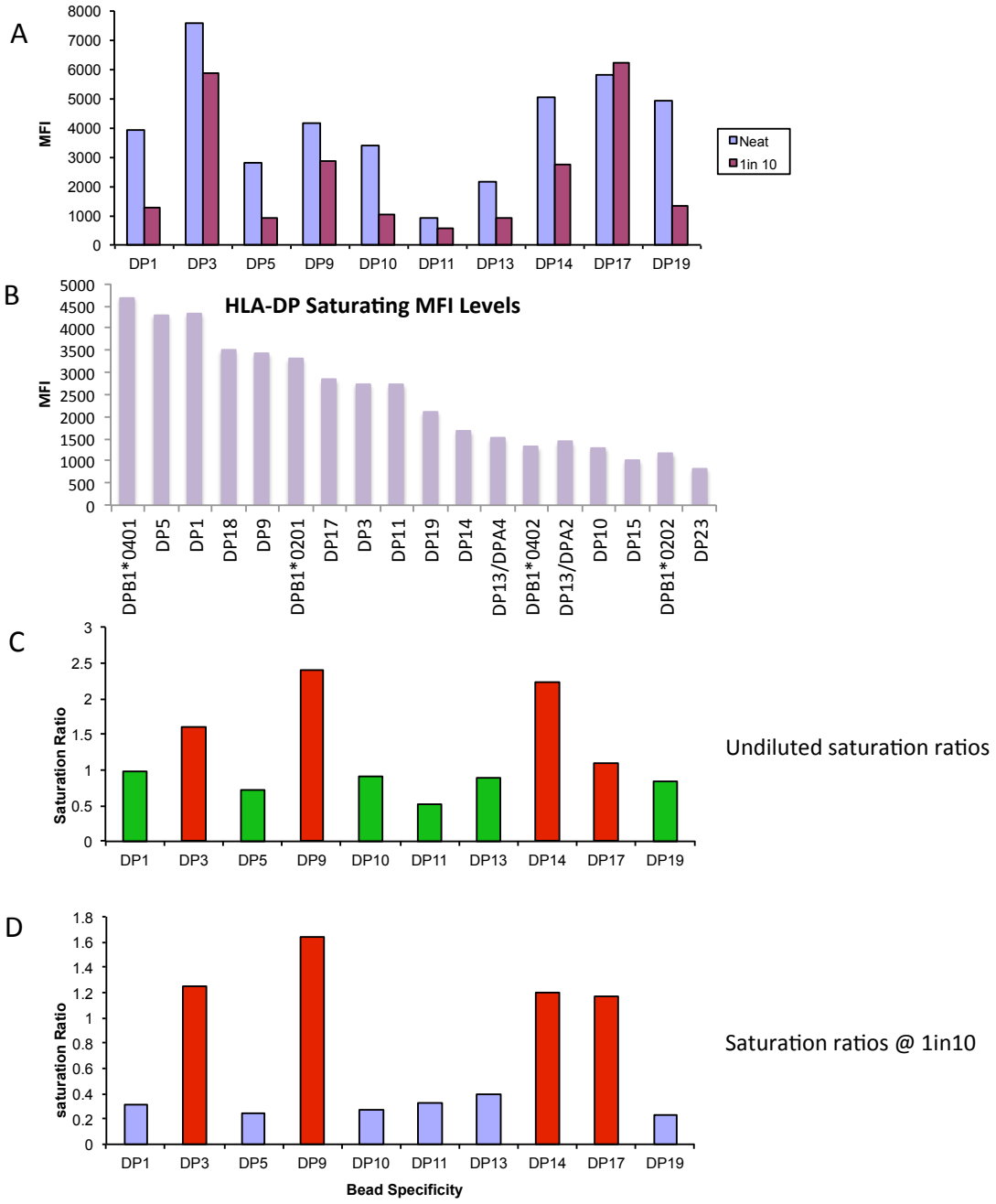


Figure 3.6: The use of saturation ratios to define epitope specific reactivity patterns. A) Positive HLA-DP specific reactions with undiluted and 1 in 10 diluted serum broadly corresponding to the 84-87 DEAV epitope. B) Levels at which the HLA-DP specific beads saturate using a HLA-DP specific monoclonal antibody. C) Saturation ratios of the serum from 3.6A shows the emergence of a 55-57 DED motif. D) The 55-57 DED epitope is clearly indicated when the sample is diluted thus correcting for the HDHE.

3.4 DISCUSSION.

The use of single recombinant HLA antigen coated polystyrene microbeads has revolutionised the way in which the transplant laboratory is able to monitor the HLA antibody status of transplant recipients. Fulfilling the early promise of the technology, daily monitoring of patient serum is now possible without the use of cumbersome crossmatching methods [91, 116]. Through analysis of the DSA and TPA profiles of a series of patients transplanted as part of HLA antibody incompatible programme we have been able to identify distinct MFI values that correspond with both the pre-transplant crossmatch results and the likelihood of rejection post-transplant [91, 116]. The determination of such cut-off values relies heavily on the correct analysis and interpretation of the readouts obtained from the microbead assay, addressing in particular the points outlined in this study.

3.4.1 Maintaining binding capacity within the assay- avoiding the high dose hook effect.

When monitoring patient antibody levels intensely, particularly in the early post transplant period, it is of vital importance to identify the points at which the binding capacity of the assay has been exceeded. In order to avoid the high dose hook effect it is absolutely crucial to maintain daily MFI readouts at a level which remains within the linear region of the binding curve. The most effective way to manage this is to apply a suitable dilution factor to the patient serum (see figure 3.2). This dilution factor may need to be adjusted through the clinical course, for example a continual sustained increase in DSA titre may necessitate a further increase in dilution factor. This is demonstrated by case 069 in our HLA incompatible transplant program who displayed a

HLA-DR9 DSA which was measurable at 1in10 dilution up until day 7 post transplant whereafter a dilution of 1in100 was required to effectively measure any further changes in DSA titre (figure 3.7).

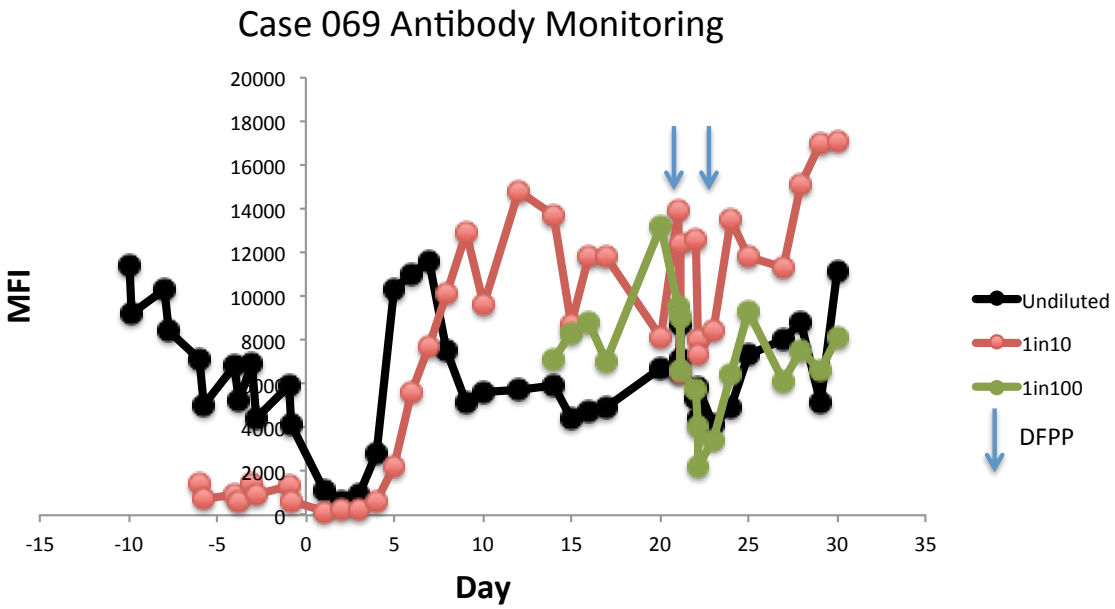


Figure 3.7: Monitoring of DSA at three dilutions. Sustained rapid rises in DSA levels are undetectable from day 7 onwards unless serum is diluted. Further dilution to 1 in 100 was required to accurately determine decline in antibody around day 24 post-transplant.

This suggests that the construction of a standard curve for antibody binding is required for each patient transplanted in our scheme to provide an indication prior to pre-transplant antibody reduction therapy as to the parameters required for accurate daily antibody monitoring. In those patients whose post transplant antibody titres exceed those measured pre-treatment it may be necessary to construct new binding curves based on these higher level samples.

3.4.2 Correcting for variation in antigen density.

The variation in antigen density on the microbeads can have major implications when comparing changes in titre levels between DSA and TPA. As shown in figure 3.5 the use of uncorrected antibody levels can give misleading results. It is of great importance when monitoring patient antibody profiles to be able to distinguish a donor specific response from that of third party. For example, in figure 3.5 the uncorrected data indicates a fall in DSA immediately post-transplant. However it is only when this data is corrected for antigen density variation that it becomes truly clear that DSA fell much more rapidly than TPA.

Corrected data is also a useful way of maintaining continuity when the bead manufacturer's change kit batches during important phases of patient monitoring. By standardising the MFI values to allow for variation in antigen density accurate patient monitoring could continue despite the change in kit batch.

3.4.3 Saturation ratios to confirm HLA-DP antibody specificity.

Due to the unique nature of the polymorphism of HLA-DP locus, saturation ratios provide a novel method of determining individual specificities in the presence of other confounding specificities. There are currently five patients in our program whose sole DSA is directed against the HLA-DP locus. By comparing the saturation ratios we are able to correlate the putative amino acid motif against which the DSA is directed. In these instances the saturation ratio can be monitored closely and changes in DSA can be distinguished from those of TPA at the HLA-DP region.

Despite the undoubted advantages given by single antigen beads, problems may arise during testing that, if not compensated for thoroughly, may give misleading results

which could potentially impact on patient care. The use of single recombinant HLA coated microbeads and the constantly evolving use of the data provided by this technology has allowed us to constantly refine our transplant protocols and pre-emptive therapy strategies.

Chapter 4

EPITOPE SPECIFIC IDENTIFICATION OF HLA-SPECIFIC ANTIBODIES

4.1 INTRODUCTION

The presence of human leukocyte antigen (HLA) specific antibodies represents one of the major obstacles to successful solid organ transplantation. Recent advances in laboratory testing, most notably the introduction of microbeads coated with single HLA antigens has allowed us to define HLA antibody specificities to new levels. However, many of the anti-HLA reactivity patterns seen in patient allosera are highly complex and require skillful interpretation from the laboratory scientist. This is particularly significant when we consider the mechanisms by which antibodies interact with their target antigen. HLA-specific antibodies recognize small clusters of non-self amino acid residues, which form epitopes. Specific epitopes are shared amongst many different HLA antigens, such that a given HLA protein can express many different immunogenic epitopes and potentially any of these epitopes, if not present on self-HLA, can illicit a humoral response. The *in silico* analysis of epitope reactivity has been possible with the use of the HLA Matchmaker algorithm [81, 82, 146] which can predict potential immunogenicity between HLA types by comparing the differences between individuals in terms of distribution of epitopes. Recent studies using mouse monoclonal antibodies and adsorption with single antigen cell lines has lead to the elucidation of over 100 immunogenic epitopes on HLA Class I [80, 147]. However, this approach is costly and time consuming.

As the methods for synthesising large repertoires of highly purified HLA proteins improve, they can be used in other modes than coupled to microbeads. One new application would be to better define the specificities of HLA antibodies. An HLA molecule may be capable of stimulating the production of multiple HLA specific antibodies, and an antibody directed against an epitope on one HLA protein should react

with the same epitope on other HLA proteins. In addition, a common problem encountered when using single antigen microbeads, are false positive reactions caused by antibody binding to denatured antigen found on the bead. By using an intact HLA protein molecule to inhibit antibody binding to native HLA epitopes we can recognize those positive reactions likely to be due to binding to denatured antigen.

Described here is a method of identifying multiple epitope specific antibodies within allosera by inhibiting the sera with selected specificities of soluble HLA (sHLA) then analyzing the remaining reactivity by microbead analysis.

4.2 MATERIALS AND METHODS

4.2.1 sHLA Production

To create soluble class I HLA protein a modified cDNA of HLA-class I alpha chain was cloned into a mammalian expression vector pcDNA3.1(-) that contained a geneticin resistance cassette. Class I HLA deficient cell line 721.221 was transfected with the expression construct and drug resistant clones were selected in growth media containing G418 (0.8mg/ml). sHLA production was measured using a capture ELISA where W6/32 was the capture antibody and anti-β2m was the detecting antibody. sHLA producing clones were sub-cloned into 96 well plates by limiting dilution and high producing sub-clones were expanded and seeded in AccuSyst-Maximizer hollow fibre bioreactors (Biovest International). Approximately 500 mg of sHLA was harvested from each bioreactor. sHLA containing supernatant was loaded on a W6/32 immunoafffinity column and washed with 40 column volumes of 20mM phosphate buffer pH 7.4. sHLA molecules were eluted from the affinity column with 50mM

diethylamine (DEA) at pH 11.3, neutralized with 1M TRIS pH 7.0, and buffer exchanged and stored at 1 mg/ml in sterile PBS.

4.2.2 Patient Allo anti-sera

Patient sera were chosen from our panel of over 100 HLA antibody incompatible (HLAi) transplant recipients, and all showed extensive HLA-class I specific antibody reactivity by microbead analysis with complex patterns of alloreactivity. In addition a panel of sera for whom high resolution HLA typing had been performed and were positive for HLA-specific antibody were analysed to determine the number of epitopes that could be elucidated.

4.2.3 sHLA Inhibition

Sera were incubated at 22°C for 30 minutes with selected sHLA. A sHLA concentration of 0.1µg/µl was deemed optimal in terms of efficacy of inhibition and minimizing the effect of sample serum dilution (see section 4.3). An identical reference sample was tested in parallel diluted to the same degree by using PBS instead of sHLA preparation. Single antigen bead analysis was carried out using Labscreen single antigen beads (LS1A04, One Lambda, Inc, Canoga Park, CA) in accordance with the manufacturers instructions. Data generated from the LABScan 100 Luminex platform were analysed using Excel spreadsheets taking trimmed mean fluorescence values from the output (.csv) files produced by the luminex analyser. Data were adjusted for background noise by subtraction of the negative control bead. Normalisation of the antigenic load for each bead was not required for this analysis as inhibition of mean

fluorescence intensity (MFI) signals were calculated as a percentage of the uninhibited MFI value.

The patterns of reactivity of all beads where initial MFI values were reduced by >80% following soluble inhibition were compared with those listed for epitope specific reactivity by El-Awar et al [80, 147], and where these corresponded epitope specific reactivity was determined.

Figures 4.1, 4.2, and 4.3 show a summary of the epitopes and the corresponding beads on which they are expressed derived from El-Awar[79, 80] et al. These maps are used to aid the definition of epitope specificity. Note that some epitopes (e.g 253Q) specificities are present on antigens of more than one HLA-locus and are therefore listed on multiple epitope maps.

Results for soluble inhibition are given as a percentage absorption for each antibody using the following formula:

$$\textit{Percentage Absorption} = \frac{\textit{Raw MFI following sHLA Inhibition}}{\textit{Raw MFI diluted reference sample}} \times 100$$

Positions of epitopes are shown using Cn3D Viewer software using the three-dimensional structure of the HLA-A02:01 molecule (accessed 13th July 2012 from http://www.ncbi.nlm.nih.gov).

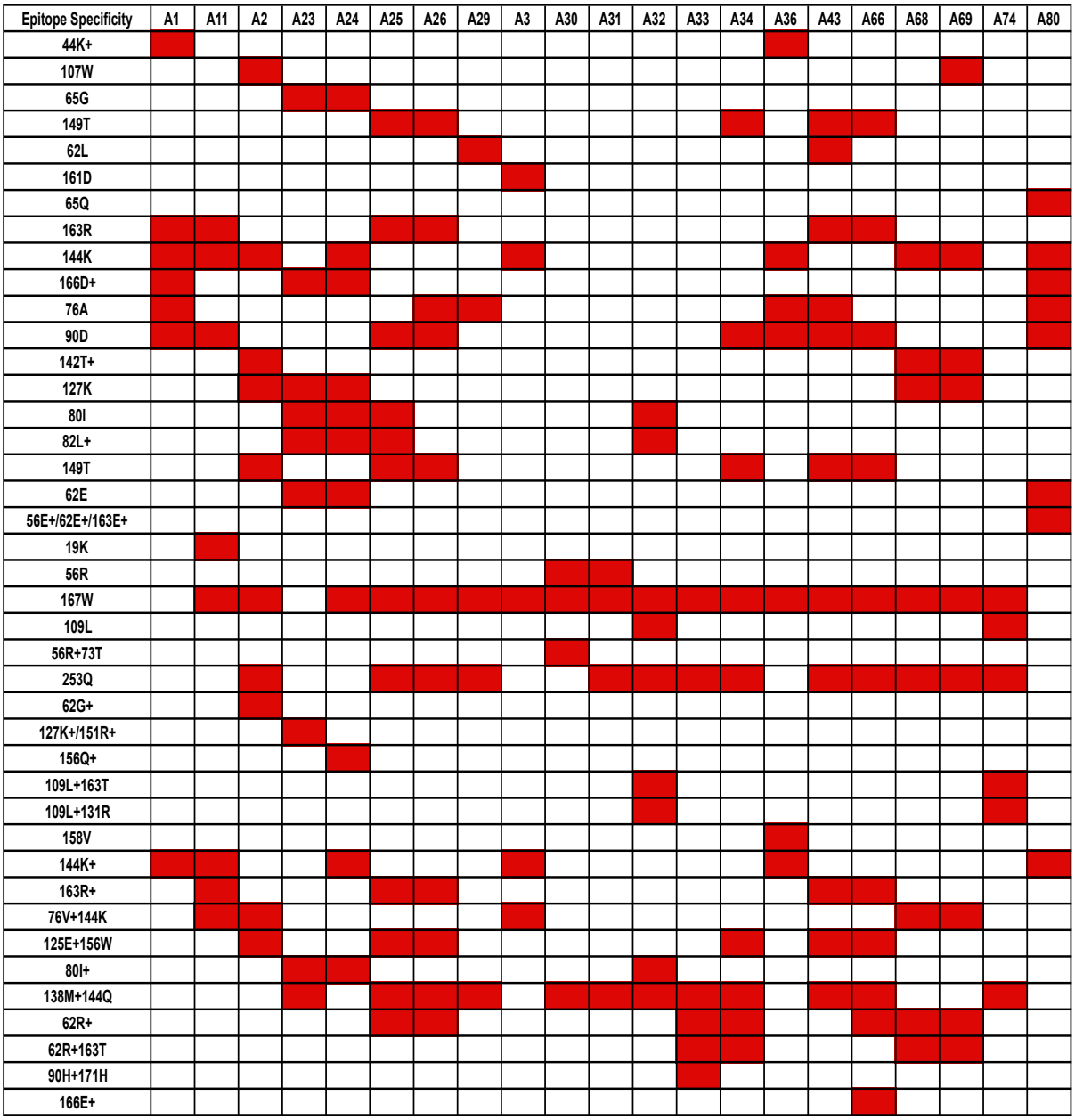


Figure 4.1: List of HLA-A locus epitope specificities and the broad HLA antigen specificities on which they are expressed.

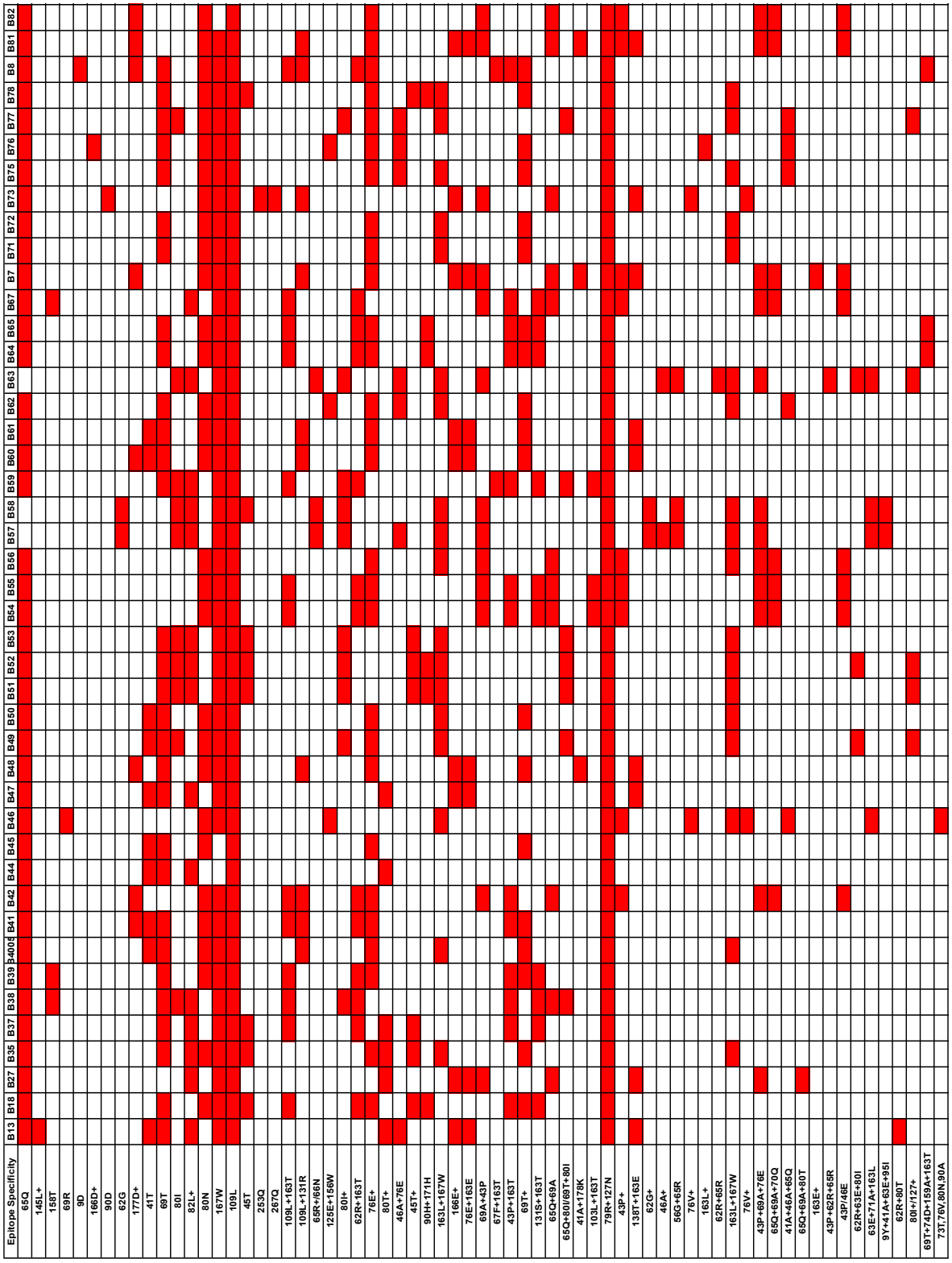


Figure 4.2: List of HLA-B locus epitope specificities and the broad HLA antigen specificities on which they are expressed.

Epitope Specificity	CW1	CW2	CW4	CW5	CW6	CW7	CW8	CW10	CW12	CW14	CW15	CW16	CW17	CW18	CW9
167W	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
194L						■									
253Q						■							■		
21H		■						■			■				■
177K				■			■								
267Q						■							■		
166E+		■											■		
103L+163T	■		■	■	■	■	■		■	■	■	■		■	
77N+80K		■	■	■	■						■		■	■	
163L+167W								■							■
76V+	■					■	■	■	■	■		■			■
73T,76V,80N,90A	■						■	■		■		■			■

Figure 4.3: List of HLA-Cw locus epitope specificities and the broad HLA antigen specificities on which they are expressed.

4.2.4 Definition of bead and sHLA specificities.

The use of the correct nomenclature makes describing the sHLA specificites and the bead specificities difficult in this context. For example the sHLA that is the product of the HLA-A*02:01 gene is too cumbersome a description to be used repeatedly and therefore will be referred to as HLA-A02:01 only. Similarly, the HLA microbead which carries the protein encoded by the HLA-A*66:02 gene will henceforth be referred to as HLA-A66:02 and so on. These differences are important to clarify as this study is concerned primarily with the protein and not the genes from which they are encoded.

4.3 RESULTS

4.3.1 Optimisation of Soluble Inhibition Conditions

Patient 065 was selected for determination of optimum soluble inhibition condition. This patient had significant microbead activity (7000-11000MFI) with the

following single antigen bead specificities; HLA-A66:02, B07:02, B13:01, B13:02, B27:05, B27:08, B47:01, B48:01, B40:01, B40:02, B73:01, B81:01. Analysis of previously published HLA class I epitopes [147], and the use of HLA Matchmaker indicated that this pattern of reactivity was due to antibody recognition of a single epitope designated 163E+166E/163E+167W, where '/' represents the possible alternative sites that may be responsible for antibody recognition. Therefore, HLA-B07:02 soluble HLA protein was added at a range of concentrations with the aim of inhibiting reactivity against all beads carrying the correct epitope. Figure 4.4a shows the reduction in MFI levels for all beads carrying the 163E+166E/163E+167W epitope, where more than one bead is specific for the same broad HLA antigen the mean of the combined MFI values was used. Effective inhibition was seen across the range of concentrations, 0.1µg/µl was chosen as a reduction of over 80% from starting MFI was observed across all beads. To support this initial observation that the pattern of microbead reactivity was due to the recognition of a single defined epitope, the experiment was repeated using an alternative sHLA specificity, HLA-B13:01, which should also absorb this antibody specificity. To rule out the possibility that our sHLA specificities were somehow pan-reactive, we also ran the experiment using a third party sHLA specificity, HLA-A02:01, which does not carry the 163E+166E/163E+167W epitope. Figures 4.4b and 4.4c show the results of this analysis. Inhibition with sHLA-B13:01 shows epitope specific reduction of reactivity comparable to that of sHLA-B7 confirming earlier observations. sHLA-A2 inhibition did not result in reduction of binding reactivity to any microbeads specific for the 163E+166E/163E+167W epitope thus confirming the specificity of inhibition.

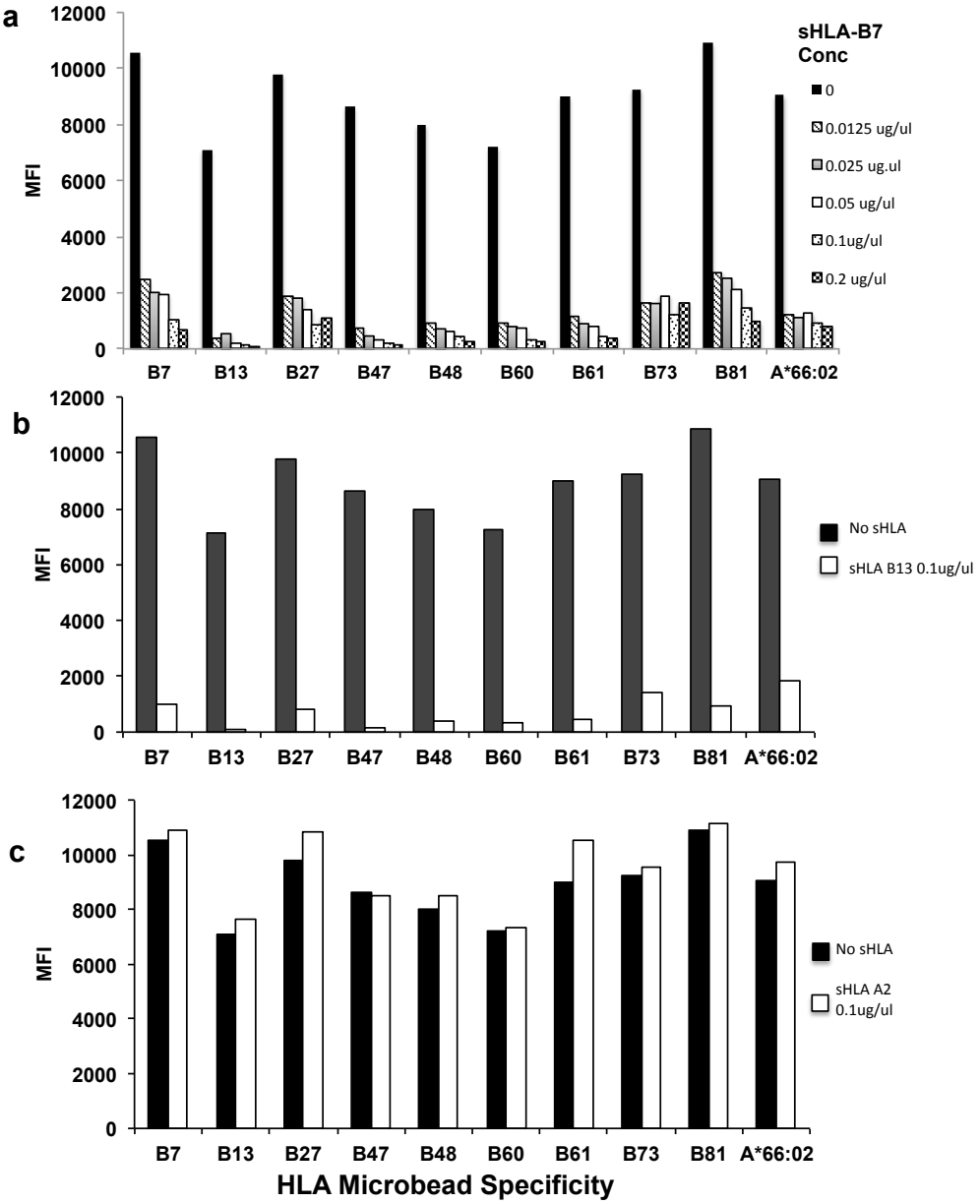


Figure 4.4: Optimisation and key examples of the epitope specific inhibition protocol. a) Determination of optimum concentration of sHLA required for effective depletion of epitope specific reactivity. b) Specific depletion of 163E+166E/163E+167W epitope specific reactivity using sHLA-B1301 protein. c) Lack of depletion following inhibition with sHLA-A0201 which does not express the 163E+166E/163E+167W epitope specificity.

4.3.2 HLA-A2 Epitope Analysis

Next, we sought to investigate the epitope composition of a number of allosera that were highly reactive against HLA-A2. There are a number of highly immunogenic epitopes that have been elucidated recently that correspond to the original cross-reactive groups (CREGS) that were originally identified by serological analysis [148-150]. The most common examples are reactivity to HLA-A2, B57, and B58 which is due to the recognition of a glycine (G) residue at position 62, and the 127 lysine (K) substitution shared between HLA-A2,23,24,68,69. There are a number of other HLA-A2 associated epitopes that will be discussed further later.

Three HLA-A2 reactive patient sera taken from waiting list patients were first inhibited using sHLA-A2 to determine the extent of HLA-A2 associated epitope reactivity. The HLA specificities substantially inhibited (>75% reduction in MFI) for each patient are listed in table 4.1.

Table 4.1: Single antigen bead specificities for each patient significantly inhibited by sHLA-A2.

Patient	Antibody Specificities inhibited by sHLA-A2
LT66	A2, B57, B58
LT68	A2, B57, B58
LT79	A2, A69, B57, B58

Epitope specific inhibition of sera from patient LT66 using soluble HLA-B57:01 shows >90% reduction in HLA-B57/58 reactivity and 60% reduction in HLA-A2 bead

reactivity (figure 4.5). This reveals the presence of antibody specific for 62G epitope (HLA-A2/B57/B58). The residual A2 reactivity is most likely due to antibody directed against the epitope listed as 43Q/62G/62G+66K/62G+76V/62G+79G by El-Awar et al [147]. The position of the 62G residue that is crucial in both of these epitopes on the HLA-A02:01 protein is also shown (figure 4.5, inset).

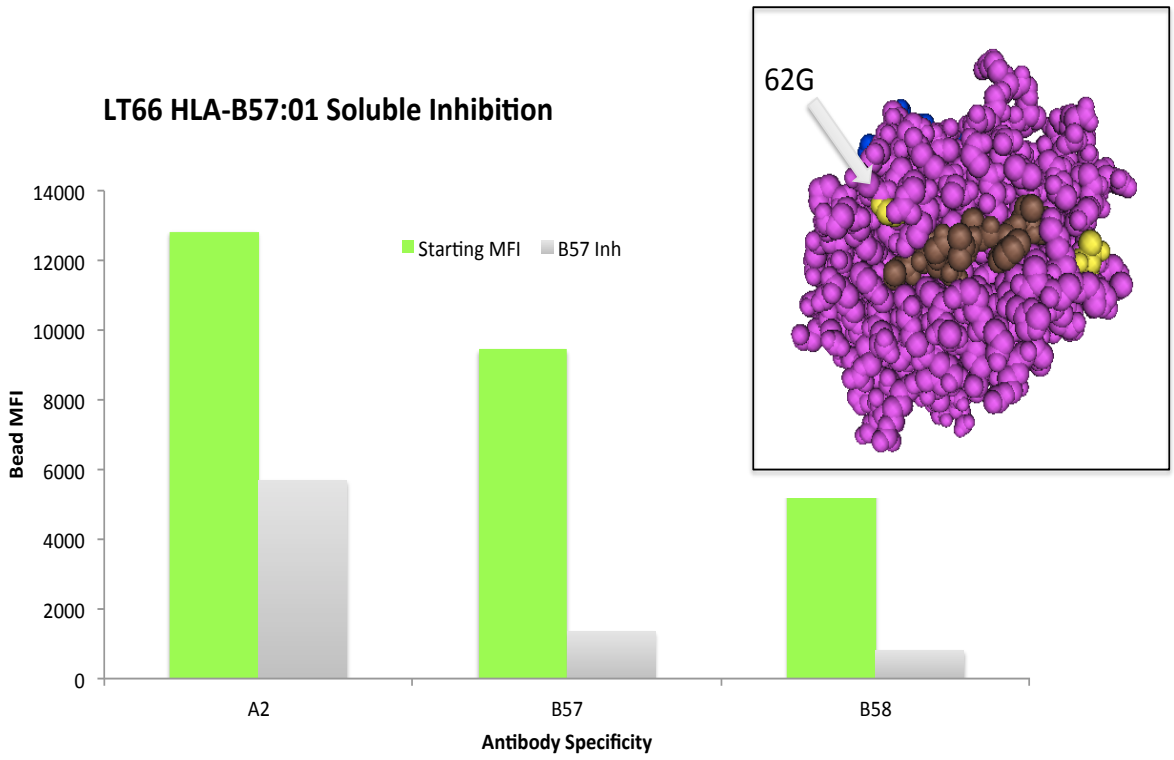


Figure 4.5: Epitope specific inhibition of sera from patient LT66. Epitope specific inhibition of sera from patient LT66 using soluble HLA-B57:01 reveals the presence of two distinct epitope specific antibodies both focussed upon the 62G polymorphic residue (inset).

The initial reaction pattern of sHLA-A2 inhibition for patient LT68 was identical to LT66, however figure 4.6 clearly shows that specific inhibition using sHLA-B57 was enough to eliminate all reactivity allowing epitope specificity to be pinpointed toward a single 62G specific antibody.

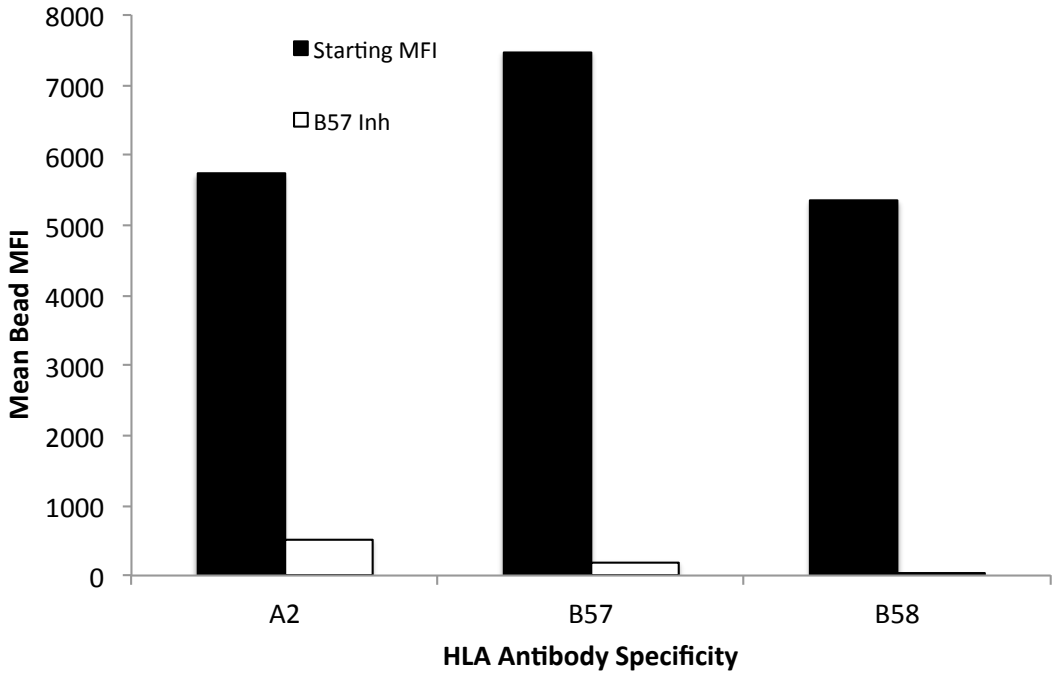


Figure 4.6: LT68- Epitope specific inhibition. Epitope specific inhibition using soluble HLA-B*57:01 shows almost 100% reduction in HLA-B57/58 reactivity and >90% reduction in HLA-A2 bead reactivity suggesting a single 62G epitope specific antibody.

Soluble Inhibition (sInh) of LT79 using sHLA-A69 resulted in a total reduction of >90% for HLA-A69 bead specific reactivity and partial depletion of HLA-A2

reactivity suggesting that multiple epitope specific antibodies are present (figure 4.7). HLA-A69 and A2 depletion can be attributed to antibody binding to a tryptophan residue (W) at position 107, which is not present on HLA-B57 and B58, hence the lack of reduction following sHLA-A69 treatment. SI with HLA-B57 reduced HLA-B57 and 58 reactivity substantially and gave a slight reduction in HLA-A2 MFI. This indicates the probable presence of both 62G (HLA-A2,57,58) and 43Q/62G/62G+66K/62G+76V/62G+79G (HLA-A2 only) specific antibodies. The latter appears to be the dominant epitope specific antibody population in this allosera mix.

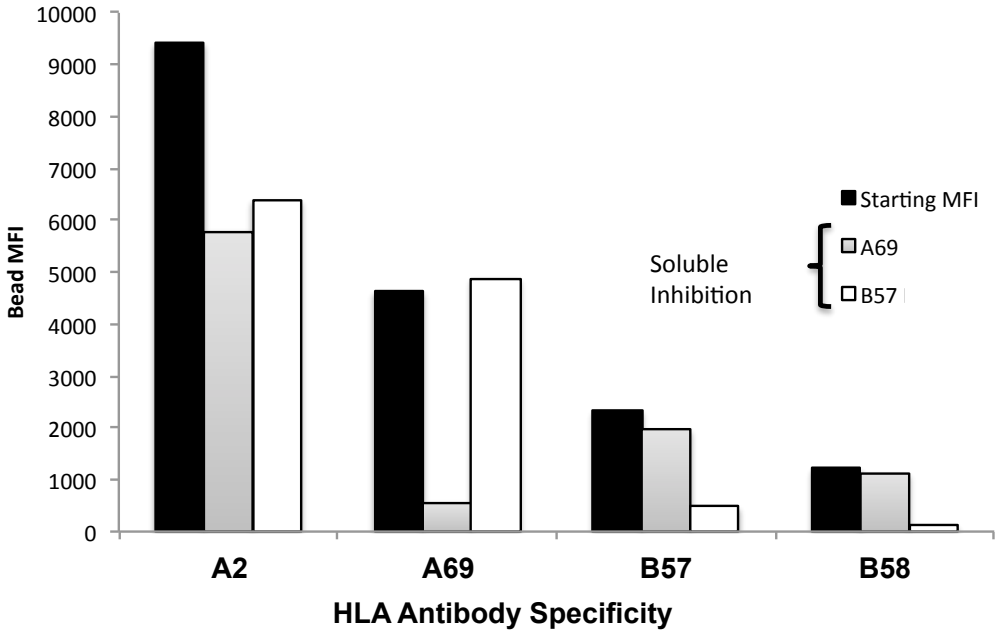


Figure 4.7: Soluble Inhibition of LT79 antibody using sHLA-A69. Total reduction of HLA-A69 specific reactivity and partial depletion of HLA-A2 reactivity suggests multiple epitope specific antibodies presents, sHLA-B57 reduced HLA-B57,58 reactivity substantially and gives a slight reduction in HLA-A2 MFI.

4.3.3 Class I Epitope Determination

A panel of class I HLA-specific antibody positive sera were characterised by luminex single antigen bead assay, then based upon these antibody profiles soluble inhibition analysis was carried out to determine the number of epitopes that could be elucidated in each case. Table 4.2 shows the individual cases, the epitope specific antibodies that could be identified, and the specificity of the sHLA used to determine the identified epitopes.

In the eight sera tested, twelve distinct patterns of epitope specific reactivity could be identified. In each case there were a number of reactions that could not be accounted for using the present repertoire of recognised epitopes. Potential reasons for this are discussed in section 4.4.

Serum	*sHLA**	*HLA Specificity of Depleted Microbead Reactions***	*Epitope Identified****	*Unexplained Bead Reactivities*
OZ14	A*02:01 Cw*05:01, 06:02, 17:01	A2 Cw2,4,5,6,15,17,18	43Q/62G/62G+66K/62G+76V/62G+79G 76V+80N	B75
QY103	A*02:01 B*07:02, 13:01	A2,24,68,69 A*66:02, B7,13,27,47,60,61,73,81	127K 163E+166E/163E+167W	B51,72 Cw1,9,10
TF40	A*24:02	A23,24	65G	Cw8
NU39	A23:01, B*44:02	A23,24,25,32 + All Bw4 associated B locus Specificities	82L/83R	None
UF4	A24:02	A23,24,80	62E	B76,82
JD51	A24:02 A29:02	A23,24 A29,43	65G 62L	A3,11,19,43 B42,56
HP117	B73:01, Cw07:01	B46,73 Cw1,6,7,10,12,14,16,18	76V+80N/73T+76V+79R	A1,11,19,28 Multiple B locus specificities
VR114	B*08:01 A29:02	B8 A29,43	9D 62L	A1,3,28,80 B52,55,56,64,65,76

Table 4.2: HLA class I epitopes identified by soluble HLA inhibition analysis.

*Specificities of sHLA used to inhibit microbead reactivity. **Those microbead reactions that were significantly depleted following inhibition. ***Epitopes matching the reactivity pattern of depleted reactions named in accordance with El-Awar et al [78, 145]. **** Those bead specificities that gave positive rections but did not fit with any known pattern of epitope specific reactivity.

4.4 DISCUSSION

The use of purified intact soluble HLA proteins can be extremely useful in gaining insight into the complex nature of HLA-specific antibody recognition. The use of soluble inhibition in this study has highlighted the epitope specific nature of the anti-HLA response and has supported previous work that demonstrated how patterns of reactivity in the single antigen bead assay can be attributed to distinct patterns of amino acid substitutions [80, 147]. Soluble inhibition can also be used to support *in silico* analysis of the mismatches between potential donor and recipient pairs [81]. Antibodies specific for potential epitope mismatches between donor and recipient can be assayed directly by soluble inhibition analysis simply by choosing one or more sHLA sharing the mismatched epitope, and depleting the reactivity of the patients' HLA-specific antibody profile accordingly.

The analysis of HLA-A2 specific reactivity has indicated that the humoral response to HLA-A2 is highly complex and often individuals will produce antibodies to more than one epitope specific to the HLA-A2 protein. This is likely to have clinical implications, in particular in those cases where MFI reactivities are being monitored post-transplant. This data shows that the reported MFI values are often a sum of two or more antibodies competing for binding sites located very closely together on the same HLA molecule. It is entirely feasible that these competing antibodies will differ not only in their specificity, but also in affinity, and functional properties (complement, non-complement fixing). This competition may result in inhibition of binding and false low MFI values (the high-dose hook effect). In such cases it may be prudent, once epitope specificities have been identified, to monitor other bead specificities that carry one or other of the HLA-A2 epitopes individually. For example if we were to consider patient LT79, monitoring the changes in MFI of the HLA-A2 specific beads may not be the

best indicator of changes in antibody titre as there are at least three separate epitope specific antibodies competing for tightly clustered epitopes on the same molecule (Figure 4.7). By analysing the changes in the HLA-A69 bead specific for the 107W epitope and the HLA-B57 or B58 beads specific for the 62G epitope a much clearer picture of antibody level changes may emerge.

The nature of epitope specific binding and the fact that in a high proportion of cases there appears to be multiple antibodies binding to the same HLA molecules may have more fundamental implications. The ability of a given serum to activate complement via the classical pathway may not necessarily only require there to be sufficient levels of suitable HLA-specific IgG, but may also be reliant on the composition of the antibodies in the serum. A mixture of separate antibody populations specific for different epitopes on the same molecule may potentially be a very potent activator of complement via the classical pathway as this mechanism requires the co-localisation of IgG molecules such that C1q can be incorporated to form the first stage in complement activation. Given that in HLA incompatible transplantation, complement dependant cytotoxic crossmatch (CDC) positive transplants have much higher rates of antibody mediated rejection [91, 116] a more detailed understanding of the factors determining serum cytotoxicity would be of great clinical benefit.

Analysis of further samples allowed the identification of 12 specific epitopes from eight sera (table 4.2). This did not account for the total repertoire of HLA-reactive microbeads in each case, and many positive reactions were unexplained following sHLA inhibition. There are a number of possible reasons for this; firstly, the panel of sHLA specificities, although extensive, may not be broad enough to cover all epitopes at this stage. Secondly, antibody reactivity specific for any antigen may be a composite of many different epitope specific antibodies. The extensive sharing of epitopes between different HLA proteins can make the potential positive identification of

individual epitopes very difficult in complex sera such as those where there is likely to be high numbers of individual epitope specific antibodies in the same serum. Thirdly, there is a growing body of evidence which shows that many of the 'positive' reactions seen in the microbead assay are false and that the antibody profiles indicated are due in large part to the non-specific binding of serum immunoglobulin to denatured HLA antigen [126, 127]. These studies suggest that antibodies direct against denatured HLA or 'crytpic' epitopes are not likely to be significant to transplantation. Therefore the identification of these non-HLA antibodies is crucial, a prospective study using sHLA to determine 'true positives' in the single antigen microbead assay is indicated.

In summary, by using soluble HLA in the standard single antigen microbead assay it is often possible to identify the specific epitopes against which HLA-specific antibody is produced. Also, antibody to very common HLA antigens are often composed of a mixture of more than one epitope specific antibody and that this complexity may be crucial both to the accurate interpretation of MFI titre levels but also to the understanding of factors governing serum cytotoxicity. Finally, sHLA inhibition may help to address the problem of recognising antibodies directed against denatured HLA antigen.

Chapter 5

EPITOPE SPECIFIC CLASS I HLA-SPECIFIC ANTIBODY QUANTIFICATION

5.1 INTRODUCTION

The recent advances in HLA antibody detection methodology, most notably the recent prominence of bead based assays, has provided the clinical transplant laboratory with valuable tools with which to rapidly and accurately monitor the changes in HLA specific antibody levels [114, 115]. The recent increase in centres with active HLA antibody incompatible (HLAi) transplant programmes has come about, at least in part, due to the emergence of these new assays[91, 116]. Indeed in our experience the data provided by these assays is vitally important in the clinical management of these transplants with changes in donor specific anti-HLA antibody (DSA) levels often preceeding the clinical signs of antibody mediated rejection (AMR) [91, 116].

The one major drawback of these assays however, is the fact they provide semi-qualitative data only. The output of these assays is given only as units of detectable fluorescence, usually referred to in the literature as mean fluorescence intensity (MFI). At best these MFI values can be considered as comparative; they enable the change in antibody levels relative to previous antibody levels to be estimated. MFI values given by these assays can be sum totals of more than one antibody which may recognise separate epitope determinants co-expressed on the same recombinant HLA molecule, as shown in chapter 4. In practice this may often mean that MFI values given for a particular antibody reactivity may be over-estimated. Conversely, these assays may often ‘saturate’ at higher levels of antibody as discussed in chapter 3. In addition, these assays are only detecting specific IgG levels, with data suggesting that presence of antibody of IgM isotype may inhibit assay sensitivity[119].

The adaptation of these widely used assays in such a way as to provide data on serum concentration of HLA specific antibody would be of clinical utility. The normal ranges of serum concentration of HLA specific antibody is currently unknown but

knowledge of this would be extremely useful particularly in understanding the requirements of antibody depletion in HLAi transplantation. Direct interpretation of MFI data and conversion to a clinically defined concentration has been hitherto absent due to the lack of an available standard reagent. The National Institute for Biological Standards and Controls (NIBSC) defines as biological standard reagent as 'a batch of a substance that has been assigned units of activity and is used as a benchmark'. Here it is shown that by using human monoclonal antibodies with HLA reactivity defined to the epitope level it is possible to derive serum concentrations of HLA-specific antibody.

5.2 METHODS

Human monoclonal antibodies (Mab) of IgG isotype were obtained from Leiden University Medical Centre. Clone SN607D8 showed single epitope reactivity 142T present on HLA-A2 and HLA-A28. Clone WK1D12 showed specificity for the 163E+166E/163E+167W epitope expressed on HLA-A*66:02, HLA-B7,B13,B27,B40,B42,B47,B48,B73,B81 and outlined in detail in chapter 4. Samples were supplied as culture supernatants, figure 5.1 shows the protein gels derived from these culture supernatants. IgG was then purified by affinity chromatography using protein G sepharose columns. Antibody was then eluted from protein G columns by the addition of 100mM glycine pH3.0, and immediately neutralised in 1M Tris pH7.0. Samples were then concentrated and dialysed into phosphate buffered saline (PBS) pH7.4. Antibodies were then quantified using a bicinchoninic acid (BCA) protein quantification assay (ThermoFisher, USA).

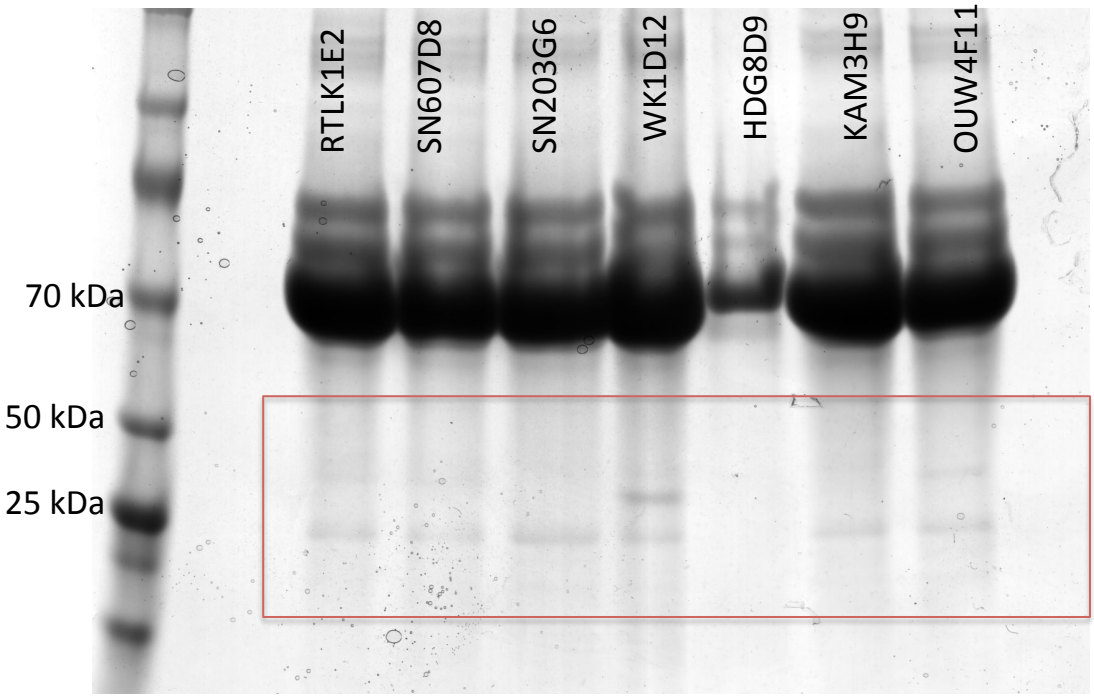


Figure 5.1: Protein gel showing HLA-specific IgG monoclonal antibody preparations run in reducing conditions. The IgG heavy chain (45 kDa) and light chain (23 kDa) bands can be seen in the boxed area. Human serum albumin (66-67kDa) are the prominent serum proteins in each sample**.** A protein marker ladder is shown on the left-hand side (refer to figure 2.9).

Each monoclonal antibody was then 'spiked' into pooled AB serum previously shown to be negative for HLA-specific antibody (data not shown) by single antigen bead assay (OneLambda Inc, CA, USA). A dynamic concentration range of 0.1-200µg/ml was used to construct a standard curve of HLA reactivity using single antigen beads. Bead MFI values corresponding to the presence of epitope specific reactivity were plotted. Due to variability in the level of antigenic expression on single antigen beads, assays were standardised by assessing the HLA expression density using W6/32 pan HLA-Class I specific MAb. MFI values were normalised to the bead representing the 75th percentile as previously described in chapter 3.

Selected sera from sensitised renal transplant patients were taken and HLA specific reactivity was determined to the epitope level. This was achieved by competitive inhibition of bead assay binding using soluble HLA protein (full details given in Chapter 4). For example in patient 065 addition of HLA-B*07:01 protein at 0.05µg/µl resulted in inhibition of all specificities carrying the 163E+166E/163E+167W epitope. This was confirmed by 163E+166E/163E+167W inhibition using HLA-B*13:01 and HLA-A*66:02 soluble protein (refer to figure 4.4a-c).

Standard curves were constructed and interpreted using Prism 4 analysis software. MFI reactivity was then converted to a defined serum concentration by interpretation of MFI reactivity and comparison with standard curves.

5.3 RESULTS

Initially, HLA specificity of the MAbs was confirmed using single antigen microbead analysis. Identical epitope specific reactivity was also confirmed in two renal transplant patients using epitope specific inhibition prior to microbead analysis (outlined in chapter 4).

Standard curve of antibody reactivity for each MAb were constructed at the following concentrations: 200, 100, 50, 25, 10, 5, 1, 0.1 µg/ml. For each curve all primary epitope specific beads were used to construct the curves, the mean HLA-A2 bead reactivity for SN607D8, and HLA-B7 bead reactivity for WK1D12. The microbead assay reached a plateau or point of saturation at MAb concentration of 100µg/ml, this is consistent for both clones tested (figure 5.2).

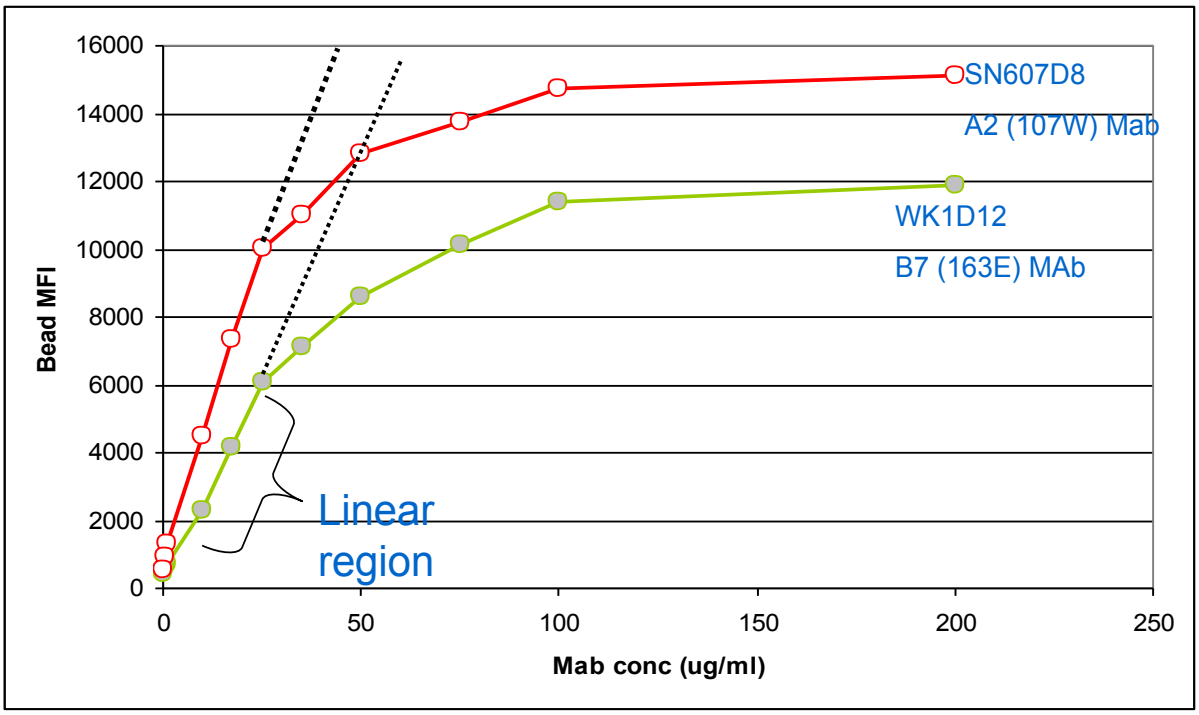


Figure 5.2: Standard concentration curves for the HLA-A2 epitope specific MAb SN607D8, and the HLA-B7 epitope specific antibody WK1D12. The linear relationship between bead MFI and antibody concentration is lost over 6000 MFI for WK1D12 and 8000 MFI for SN607D8. This implies a 'working range' of detectable antibody of up to 25-35µg/ml.

A serum sample for patient QE01 (142T epitope specific antibody) was then tested by single antigen microbead assay and the mean MFI of the A2 beads was read off to give an estimate of serum concentration. A mean A2 bead MFI of 6200 corresponded to a serum concentration of 31.7µg/ml. This patient is active on our local waiting list and consistently produces a HLA-A2 reactive MFI in the region of 5000-7000 indicating a stable antibody.

We then analysed patient 065 who received a HLA antibody incompatible kidney graft in our centre. The chosen sample was taken 17 days post-transplantation

during a period of antibody mediated rejection marked by a rapid increase in donor HLA-B7 (163E+166E/163E+167W) specific reactivity. When tested at neat dilution this sample gave an estimate of 163E+166E/163E+167W specific serum concentration of 93.1µg/ml which equated to an initial MFI reading of 10,600. However, due to the fact that 93.1µg/ml is close to the 100µg/ml estimated saturation point of the assay it was felt that this concentration gave a significant under-estimate of the true antibody level. Thus the sample was re-tested at 1in10 dilution and gave an MFI corresponding to 18.5µg/ml, equating to an overall estimated serum concentration of 185µg/ml. To test the robustness of this approach, the concentration of the original MAb (WK1D12) was adjusted to 185µg/ml and tested again with single antigen microbeads. Figure 5.3 shows that the MFI values obtained for each bead specificity was directly comparable and that any differences observed could be attributable to normal assay variation.

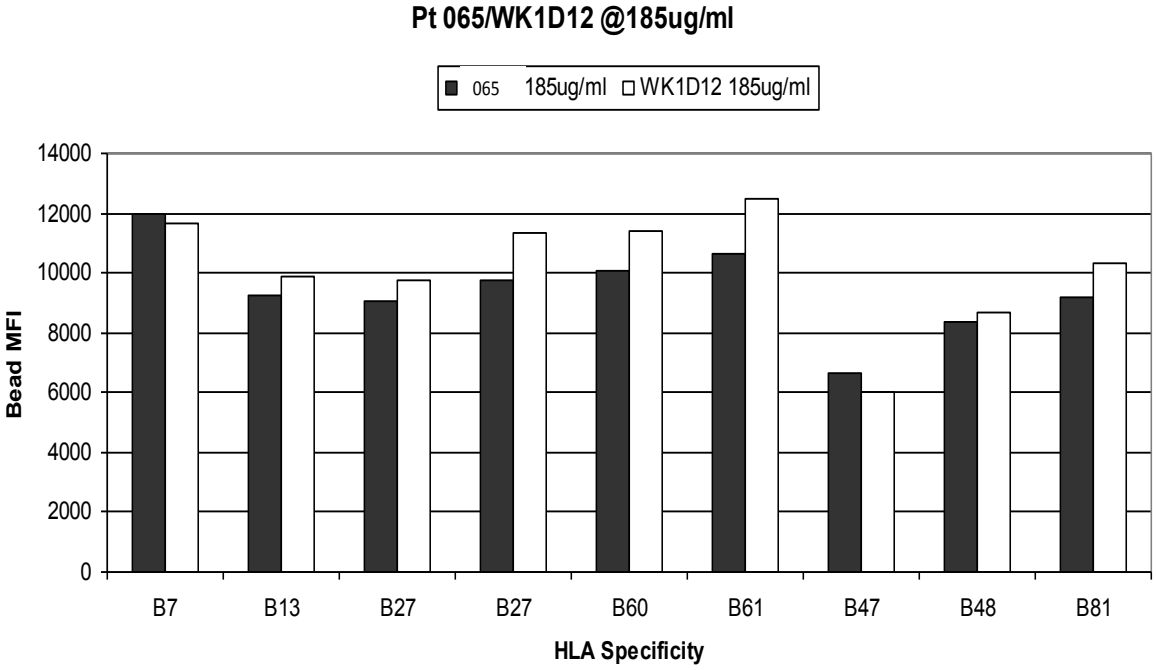


Figure 5.3: Microbead analysis of patient 065 and MAb WK1D12 with sample concentration adjusted to 185µg/ml. Serum from patient 065 was estimated to have a serum concentration of 185µg/ml, monoclonal antibody WK1D12 was adjusted to 185µg/ml also and both samples were tested by microbead analysis.

In the case of patient 065 the peak antibody concentration post-transplantation was estimated to be 185µg/ml. Samples taken from this patient at specific time-points pre and post-transplantation were retrospectively analysed and a serum antibody concentration for each specific time-point was derived. This data is given in Table 5.1 and the time-course is plotted in terms of reactive MFI and estimates of serum concentration in figure 5.4.

Table 5.1: Estimated serum concentration of 163E+166E/163E+167W specific antibody through the course of HLAi transplantation. A serum concentration of 0 denotes MFI dropping below the sensitivity threshold of the standard curve. *sample tested at 1in10 dilution and serum concentration derived by extrapolation as described.

Tx Day	HLA-B7 Bead reactive MFI	Estimated serum conc (ug/ml)
-3	2894	12.8
-1	1610	1.2
1	236	0
5	243	0
10	2511	9.3
13	3728	20.3
17	12,000	185*
24	4725	29.3
55	802	0

This analysis indicates that this approach utilising standard curves of defined epitope reactivity is sensitive down to a serum concentration in the region of 1-2µg/ml, the lower threshold of MFI reactivity which could be quantified is in the region of 800. The estimated upper limit of quantifiable MFI reactivity is in the region of 40-50µg/ml

with dilution analysis required to accurately quantify HLA-specific responses above this concentration range (figure 5.2).

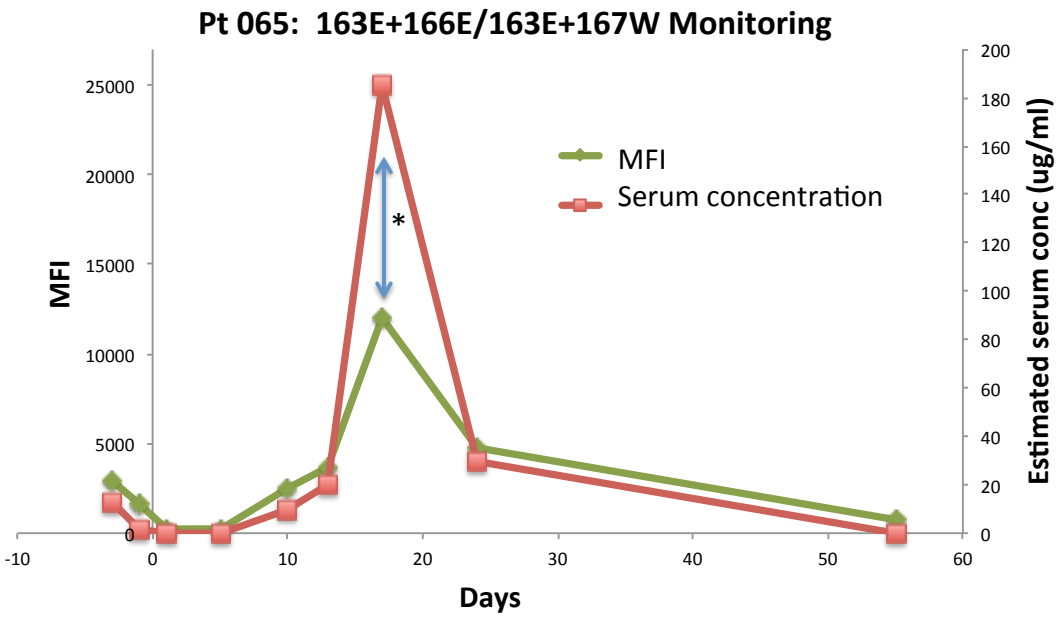


Figure 5.4: Comparison of MFI values with serum concentration of antibody. It can clearly be seen that when the antibody concentration exceeds the dynamic upper limit of the assay (days 13-24), the MFI and actual serum antibody concentration show the greatest disparity (*)

5.4 DISCUSSION

This study provides a simple method to estimate serum concentration of epitope specific anti-HLA antibodies. For the first time this gives us a valuable insight into the range of levels at which circulating anti-HLA specific antibody can be found in a patient population. The individual patient sera used here were chosen both for their epitope reactivity but also to represent different cycles of a patients' response to foreign HLA. Patient QE01 is currently active on our local kidney transplant waiting list and

consistently gives a HLA-specific MFI in the region of 5000-7000. In this study this was calculated as a HLA epitope specific serum IgG concentration of 31.7µg/ml. As this is a simplified example of single epitope reactivity we would expect the anti-HLA specific fraction of circulating IgG to typically be a multiple of this amount as many patients carry a range of HLA-specific antibodies. Based on examination of normal ranges of total IgG in serum a concentration of 31.7µg/ml would constitute around 0.05-0.2% of total circulating IgG.

Patient 065 provides an example of a dynamic antibody response in the immediate pre and post-transplantation period in HLA antibody incompatible transplantation. Analysis showed a pre-transplant HLA-specific antibody starting concentration of 12.8µg/ml, which was reduced below quantifiable level by five rounds of pre-transplant double filtration plasmapheresis (DFPP) therapy. Antibody levels rose in conjunction with an episode of biopsy proven antibody mediated rejection (AMR) around 10 days post-transplant rising to a peak antibody concentration of 185µg/ml on day 17 post-transplant. This peak response level equates to approximately 1% of the total serum IgG being specific for the donor kidney at this stage. Subsequently, levels of donor reactive antibody fell to 29.3µg/ml at post-transplant day 24, then to sub-quantifiable levels at day 55. These falls in serum antibody concentration were consistent with resolution of AMR and restoration of renal function.

Knowledge of the serum quantification of anti-HLA antibody has clinical utility when monitoring the changes in antibody titre, with particular benefit to centres supporting HLAi transplant programmes. These measurements may assist in the assignment of immunological risk prior to transplant, with the potential assignment of critical cut-off values of antibody concentration above which increased risk of AMR could be expected.

It is also important to note the limitations of this approach. At present this method allows the accurate quantification of single epitope specific antibodies. The examples given here are common epitope specific reactivities seen in a number of patients in our transplant cohort. It is not yet known the effect of multiple epitope specific antibodies in a given patient serum. Also it is labour intensive to define the exact epitope specificities present in a sample serum, requiring extensive soluble phase absorption experiments and expensive microbead analysis.

The microbead assays in use in virtually all modern transplant laboratories are themselves not well suited to this application. As noted earlier these assays are easily saturated at higher antibody concentrations (data suggests 100µg/ml), therefore it is of paramount importance to recognise when a particular sera is likely to have saturated the assay and to dilute the sample accordingly. The subsequent dilution of patient sample may then lead to a larger degree of error when converted back to pure concentration, although the data shown in figure 5.2 suggests this degree of variation is not unacceptable. Other studies have suggested that other immunoglobulin isotypes such as IgM may inhibit the accurate measurement of IgG by these assays[119] so sample pre-treatment with specific reducing agents such as dithiothreitol (DTT) may be worthy of further investigation.

Chapter 6

IgG SUBCLASS HETEROGENEITY IN HLA-SPECIFIC ANTIBODIES

6.1 INTRODUCTION

Donor HLA-specific antibodies are not always an obstacle to organ transplantation: a complete understanding of the heterogeneity of these antibodies and the circumstances which cause this to vary is therefore important in the development of strategies to safely transplant across antibody barriers. Hyperacute rejection mediated by HLA-specific antibodies seems to be dependent on a cytotoxic pretransplant crossmatch but non-cytotoxic levels of antibodies are not always associated with immediate graft failure, whether detected by flow cytometry [151, 152] or by solid-phase assays [153]. The specificity of alloantibodies in terms of the HLA isotype recognised is also a significant factor; pre-formed HLA class I-specific antibodies are considered to be more harmful to kidney transplants than those against Class II HLA [154]. Therefore both qualitative as well as quantitative factors determine the pathogenicity of HLA-reactive antibodies.

A third factor likely to determine alloantibody pathogenicity is immunoglobulin isotype. IgG is the predominant HLA-specific antibody; HLA-specific IgA is variously thought to be protective or not directly harmful to allogeneic grafts [155], and donor HLA-specific IgM is generally considered of limited, direct clinical significance [156]. There are four subclasses of IgG which are functionally distinct as a result of different heavy chain gene usage. The germ-line IgG heavy chain genes are in the order Cγ3, Cγ1, Cγ2, Cγ4 with subclass switching being sequential, in this order, and antigen driven, under T cell control. In order to evaluate the role of IgG subclasses in transplantation we must have a reliable method to detect and characterise each type of IgG specific for individual antigens in a patient's serum. Single antigen bead assays (SABA) provide a system to do this. Lobashevksy et al [157] and Honger et al [158] each used bead assays to investigate the significance of pretransplant HLA-specific IgG

subclasses but with seemingly contradictory conclusions. IgG subclass distribution is likely to influence the pathogenicity of HLA-specific sera. However, it is not known if there is a characteristic distribution of IgG subclasses for HLA specific antibodies, whether this is a property of the individual or of the antibody specificity, and if this is dependent on the type of immunising event. In this study we have optimised a standard HLA SABA for IgG subclass characterisation and describe the distribution and relative levels of IgG subclasses of 148 HLA antibody specificities in sera from highly sensitised, prospective renal transplant patients. We found a range of subclass profiles between and within individuals and this is dependent on the source of antibody stimulation.

6.2 METHODS

6.2.1 Study samples

Sera from fifty-one patients were studied and were selected primarily on the basis of their recruitment into our HLA antibody incompatible transplant (AiT) programme [91]; pretreatment sera were used. These cases are therefore characterised by a relatively high level of HLA-specific sensitisation. All sera were shown to contain donor-specific (DSA) and third party-specific (TPA) antibodies using single HLA microbead assays for class I (HLA-A/B/C) and class II (HLA-DR/DQ/DP), (One Lambda Inc, CA, USA) and analysed on a standard luminex platform (X-MAP 100). These kits use a polyclonal goat anti-human IgG-specific antibody and therefore detect total IgG binding to the HLA on each bead.

Selected antibody specificities were tested for the presence of IgG1-4 using individual HLA coated beads (Labscreen Singles, One Lambda Inc, CA, USA). The bead specificities chosen corresponded to the DSAs for each subsequent transplant plus the highest level TPA if present. There was therefore no intended bias of particular specificities. The specificities tested are listed in Table 6.1. Note that from the 148 selected specificities determined by total IgG, there were 10 specificities that were not detectable using IgG subclass-specific monoclonal antibodies. This is described further, in table 6.2.

Table 6.1: HLA specificities tested and number of sera containing each specificity. All antibodies were primarily characterised using a pan IgG-specific secondary antibody. Ten specificities were below critical threshold values using subclass-specific secondary antibodies and these are indicated by the lower numbers in brackets.

HLA Class I Bead Specificity	Number of sera
A1	7
A11	3
A2	13(12)
A24	6
A25	2
A3	5(4)
A30	1(0)
A32	1
A33	2(1)
A34	1(0)
A66	1
A68	2
B13	2
B27	1
B35	3
B44	2
B53	1
B57	4
B60	3
B62	5
B65	1
B7	6
B8	4(3)
C3	1

HLA Class II Bead Specificity	Number of sera
	1
DP2	2
DP4	2
DQ2	4
DQ4	1
DQ5	4
DQ6	4
DQ7	7(6)
DQ8	2
DQ9	1
DR1	2
DR10	1
D103	1
DR11	5
DR13	2
DR14	1
DR15	1
DR17	3
DR4	13(11)
DR52	2
DR53	1
DR7	5
DR8	3(2)
DR9	3

Table 6.2: Ten cases with subclass-specific MFI values below the pos-neg threshold of 5 times the negative bead average. (IgG3 62.7, IgG1 120.6, IgG2 72.0, IgG4 17.2). *Pan IgG MFI determined using multiple antigen beads and confounding specificities prevent an estimate of a specific MFI value.

Specificity	IgG3	IgG1	IgG2	IgG4	Pan IgG
A34	17	118	6	8	2200
DQ7	15	114	11	8	2000
A3	15	111	11	11	*
A33	58	83	16	10	*
A30	43	81	12	1	193
DR4	7	75	1	1	1300
B8	17	63	28	11	781
DR4	27	5	4	4	2700
DR8	18	7	11	4	*
A2	5	13	3	1	1600

6.2.2 HLA-specific IgG subclass assay

Assay conditions followed the manufacturers instructions in that bead to serum ratios were adhered to but the pan-IgG reactive secondary antibody was replaced by subclass specific phycoerythrin (PE) conjugated mouse monoclonal antibody (IgG1, clone 4E3; IgG2, clone 31-7-4; IgG3, clone HP6050; IgG4, HP6025, Southern Biotech, Birmingham, AL, USA). The final concentration of each subclass specific antibody was 0.25ug/ul. Negative and positive control sera were supplied by the manufacturer. Each assay was finally resuspended in 85ul luminex wash diluent for analysis on the Luminex100^{TM} analyzer. Subclass specific monoclonal antibodies were validated by coating individual microbeads with IgG subclasses purified from sera from four human myeloma patients. Analysis of reactivity patterns showed that potential cross-reactivity

between subclass specific monoclonal antibodies represents <5% for all monoclonal antibodies as shown in Table 6.3.

Table 6.3: Monoclonal antibodies binding to IgG subclass coated beads. Values are raw MFI. Specific binding levels are in shaded boxes, non-specific (cross-reaction) levels are given with percentage of specific binding in brackets. Note, no case of non-specific binding exceeds 5%.

		Bead Specificity			
Monoclonal Antibodies		IgG1	IgG2	IgG3	IgG4
4-E-3	IgG1-specific	**6950**	226 (3.13%)	14 (0.2%)	9 (0.13%)
37- 7 -4	IgG2-specific	248 (4.42%)	**5342**	10 (0.18%)	11 (0.19%)
HP6050	IgG3-specific	66 (1.7%)	38 (0.99%)	**3854**	8 (0.21%)
HP6025	IgG4-specific	37 (1.42%)	18 (0.61%)	11 (0.37%)	**2936**

The incidence of antibody specificities of each subclass were determined on the basis of a critical threshold value for each subclass. There are no existing controls or standard sera for HLA specific antibodies because of the complexity and individual nature of each HLA-specific antibody as discussed in chapter 5. We therefore used a stringent assessment of positivity relative to background binding to the negative control bead. This was set at greater than 5 times the average negative control bead mean fluorescence intensity (MFI) of all tests for each subclass. This reduces the risk of false positives at the expense of potentially increasing false negatives but is justified given that higher levels of HLA specific antibodies are likely to be clinically more significant than lower levels. Relative levels of each antibody between individuals and groups were compared using bead MFI values [91].

6.2.3 Assignment of sensitizing event to antibody specificity

Where possible, each antibody specificity was assigned a sensitizing event based on historical records, as follows. Sensitisation arising from pregnancy was assigned if the antibody specificity corresponded to the partner's mismatched HLA and if the timing of the antibody appearance was consistent with the dates of the pregnancy. Similarly, sensitisation due to a previous transplant was assigned if the antibody specificity corresponded to a graft HLA mismatch and the timing of the appearance of the antibody was consistent with rejection of the graft. Blood transfusion stimulated antibody was assigned for cases with a known transfusion history, the absence of a temporal relationship with other sensitising events containing the relevant HLA and the exclusion of specificities corresponding to any previous pregnancy or transplant mismatch. In certain cases this allowed assignment of different specificities to different sensitising events in single individuals.

6.2.4 Statistical analysis

Statistical analysis was performed using SPSS for windows, version 19.0 (SPSS Institute, Chicago, IL). Frequency differences were assessed using the Chi Squared test, and quantitative comparisons were assessed using the Wilcoxon Rank test. The level of statistical significance was set at $p<0.05$.

6.3 RESULTS

6.3.1 Detection of HLA-specific IgG subclasses

HLA-specific IgG subclasses were detected and measured in 148 separate sets of bead assays and raw MFI values recorded. Figure 6.1 shows these MFI values (y axis) for assays of all the specificities each arranged in the same order, ranked by the corresponding IgG1 level. This illustrates that the patterns of IgG2, IgG3, and IgG4 subclass detection are not due to crossreactivity with the IgG1-specific antibody as the rankings are clearly different, thus confirming earlier validation data provided in table 6.3. Linear regression analysis comparing MFI values between all the paired subclass MFI values in each set of assays gave no significant correlations. The ranked MFI values of all subclasses form a smooth curve with no obvious step between a negative and a positive threshold.

Each isotype-specific antibody gave different control bead values, so the positive threshold MFI values used for each HLA specific IgG subclass were different, as follows: IgG1 120.6, IgG2 72.0, IgG3 62.7, IgG4 17.2. In ten of the 148 antibody specificities tested, the MFI values of all subclasses were below the chosen positive threshold (details given in Table 6.2). Certain examples of these specificities have MFI levels that are less than 1x the negative control beads despite the specificity being detected using the pan-IgG antibody with single antigen beads. Others have MFI close to our threshold values but, although it is probable that these sera contain some corresponding HLA reactivity, they were excluded from the qualitative analysis as we could not determine which subclass did or did not contribute to the pan-IgG reactivity. Together these data indicate that; (1) Detection of HLA specific IgG using pan-IgG polyspecific secondary antibodies is more sensitive than using subclass-specific

monoclonal antibodies. (2) The pan-IgG assay may detect antibodies other than IgG1-4. (3) The chosen 5x negative bead value threshold is likely to exclude some specific antibodies.

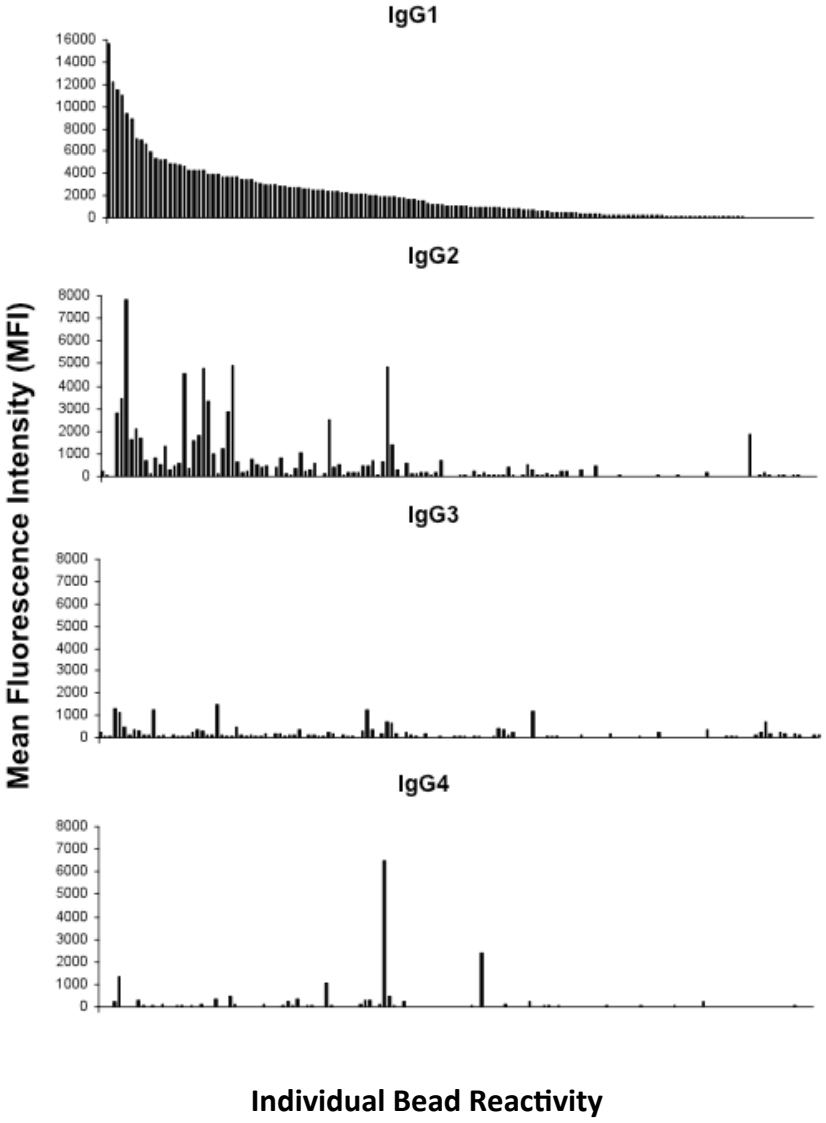


Figure 6.1: Distribution of MFI levels of all HLA specificities for the four IgG subclasses. Each vertical bar represents the result of a serum tested against one specific bead. The data in all graphs are sorted by IgG1-specific MFI level so that the position on the x axis of each serum/bead test is the same for the replicate assays using the four subclasses.

6.3.2 Subclass distribution

The term *specificity* is used to refer to the HLA on the bead against which an antibody was detected. *Antibody* refers to the particular immunoglobulin and thus we can refer to a specificity being comprised of one or more antibodies (IgG1 or IgG1 and IgG2, etc). Subclass *profile* will be used to describe the mixture of subclass antibodies for a given specificity. The frequencies of the IgG subclasses amongst the 138 HLA specificities are shown in Table 6.4.

Table 6.4: Incidence of each IgG subclass amongst 138 HLA-specific antibodies. The incidence of each IgG subclass is analysed in total, and separately for HLA class I and class II specificities.

	All Antibodies		Class I specific antibodies		Class II specific antibodies	
	n	%	n	%	n	%
IgG3	71	51.4	38	53.5	33	49.3
IgG1	126	91.3	64	92.1	62	92.5
IgG2	78	56.5	43	60.6	35	52.2
IgG4	55	39.9	30	42.3	25	37.3

All subclasses were detected but IgG1 was the most prevalent followed by IgG2, then IgG3, with IgG4 being the least. The prevalence of each subclass is similar for both HLA Class I and Class II specific antibodies. Twelve of the 138 (9%) specificities do not have IgG1 levels above our cut-off threshold. Of these twelve antibodies, eleven have IgG3 (nine exclusively), and one IgG2 only. No specificities had only IgG4. Of the 126 specificities which include IgG1, 38 have IgG1 exclusively, the remainder have a mixture of subclasses but none with IgG4 only. Most specificities (90/138) are comprised of mixtures of IgG subclasses. The distributions of these IgG subclass

profiles for the 138 specificities are shown in Figure 6.2A. This shows that for both HLA Class I and Class II specific antibodies there are two predominant IgG profiles; one restricted to IgG1, the other with all four subclasses detectable. The subclass profiles are given as the presence or absence of each subclass in the order IgG3, IgG1, IgG2, IgG4 (ie the germ-line heavy chain gene sequence).

6.3.3 Sensitising events and IgG subclasses

Unequivocal identification of sensitising events was possible in 38 of the 51 sera, allowing the cause of antibody stimulation to be assigned to 109 of the 138 specificities. In these 109 specificities, subclass MFI values were above the cut-off thresholds in 106 (as described in Table 6.1) and could therefore be analysed qualitatively as follows and shown in Table 6.5a. IgG1 was found to be the dominant subclass of HLA-specific antibodies for all causes of sensitisation. While IgG2 is second most prevalent in blood transfusion and kidney graft sensitisation antibodies (48% and 68%, respectively). Amongst the pregnancy stimulated specificities IgG3 is the second most prevalent antibody at 68% ($p=0.052$) with IgG2 at 50%. IgG4 is of low frequency in our transfusion sensitised cases (26%) compared with this antibody in the combined two other groups (47%) ($p=0.046$). Comparisons of bead MFI values as an indicator of relative antibody amount (and not dependant on an arbitrary cut-off) also show differences due to the route of sensitisation. Median MFI values were found to be higher in transplant stimulated IgG1, IgG2, and IgG4 antibodies compared with the other routes of sensitisation (statistics for all comparisons are given in Table 6.5b). The median IgG3 MFI value was highest in the pregnancy group but overall there are no significant differences in the distribution of IgG3 MFI levels between the three modes of sensitisation.

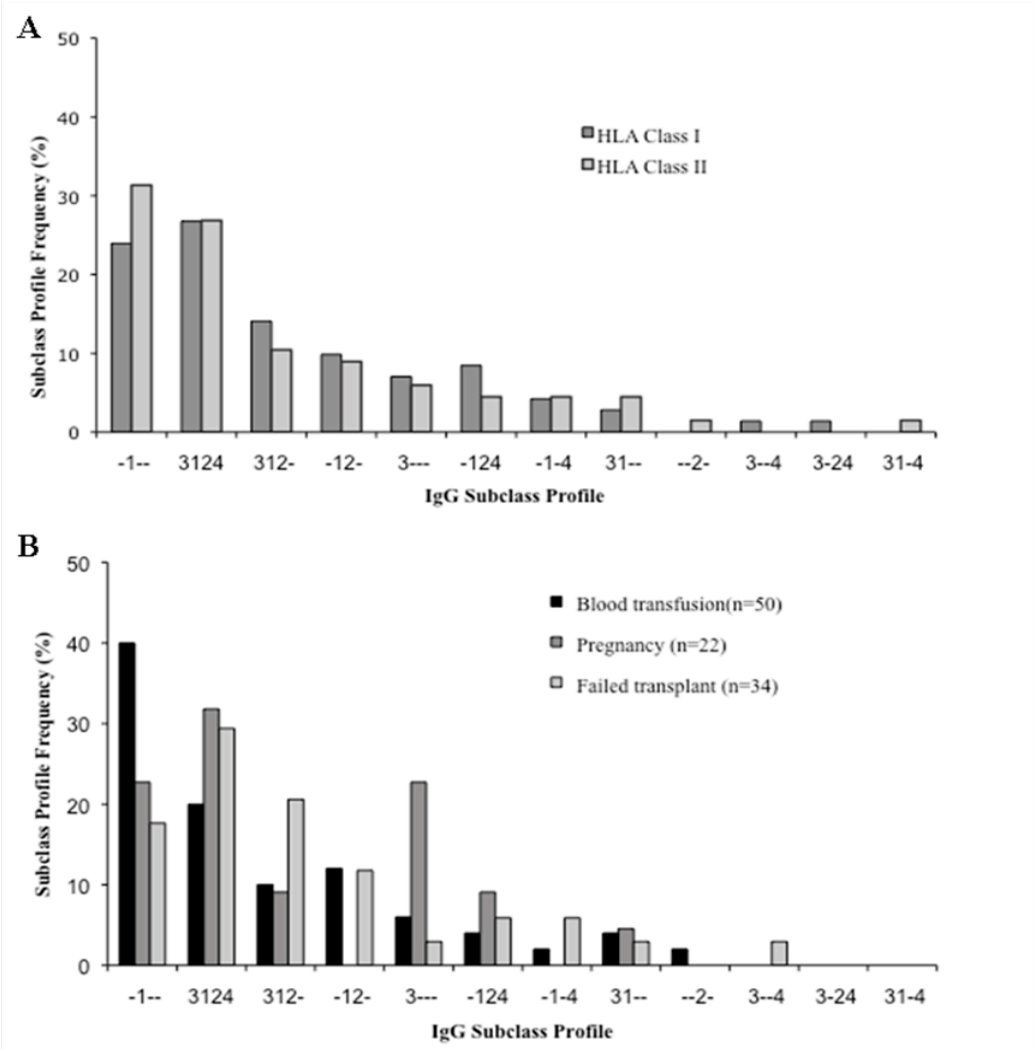


Figure 6.2: Distribution of IgG subclass profiles, shown in 138 HLA specificities indicating the presence (subclass number) or absence [-] of each subclass. The combinations of each four are given in germ-line heavy chain gene order. A) Frequency of profiles for HLA class I and Class II antibody specificities with no significant differences. B) The distribution of the profiles according to mode of sensitisation. The distribution of the profile containing IgG1 only ("-1--") is significantly different than that expected (p=0.033); the distributions of the remaining profiles do not differ from expected.

The distribution of HLA-specific IgG subclass profiles in the three sensitisation groups is shown in Figure 6.2B. This illustrates general differences associated with sensitisation route. IgG1 only dominates antibodies resulting from transfusion

(p=0.033), while for both pregnancy and failed graft sensitisation the most prevalent profile is that comprising all subclasses. For the failed transplant cases the IgG1 only profile is third in order of prevalence. Consistent with the raised frequency of IgG3 in the pregnancy cases, the IgG3 only profile is more common in this group (23% vs 8% in the non-pregnancy cases, p=0.052).

Table 6.5: a) **Incidence of each HLA-specific IgG subclass in the three sensitisation groups.** (* p=0.046 compared with the the non-transfusion cases; no other significant differences). b) Quantitative differences between type of sensitisation for each IgG subclass. Median MFI values in bold, interquartile values in brackets.

a)	Blood transfusion		Failed transplant		Pregnancy	
	n (50)	%	n (34)	%	n (22)	%
IgG3	20	40	20	59	15	68
IgG1	46	92	32	94	15	77
IgG2	24	48	23	68	11	50
IgG4	13	26*	19	47	9	41

b)	Blood transfusion	Failed transplant	Pregnancy
	n=53	n=34	n=24
IgG3	(18.0) **43.0** (123.5)[a]	(19.5) **80.0** (270.5)[b]	(29.3) **116.5** (191.75)[c]
IgG1	(216.0) **903.0** (2280.5)[d]	(510.0) **1940.0** (4238.25)[e]	(101.8) **915.5** (2755.3)[f]
IgG2	(12.0) **48.0** (301.0)[g]	(52.8) **233.5** (1888)[h]	(22.5) **113.5** (358.0)[i]
IgG4	(4.0) **9.0** (17.0)[j]	(7.0) **16.5** (40.25)[k]	(3.8) **9.0** (58.0)[l]

Statistics

IgG3	a vs b, p=0.09	a vs c, p=0.144	b vs c, p=0.7
IgG1	d vs e, p=0.071	d vs f, p=0.910	e vs f, p=0.042
IgG2	g vs h, p=0.001	g vs i, p=0.414	h vs i, p=0.016
IgG4	j vs k, p=0.052	j vs l, p=0.277	k vs l, p=0.581

We found no subclass to be associated with any particular HLA type or isotype. However, we did see a higher frequency of DQ-specific IgG4 amongst the transplant stimulated antibodies (7/8) compared with IgG4 non-DQ specificities (9/26) (χ^2=6.87,

p=0.009). In contrast to this, only 3/12 non-transplant stimulated DQ specificities contained IgG4 (compared with the 7/8 transplanted DQ specificities, χ^2=7.5, p=0.006).

These observations indicate that the route of sensitisation influences IgG heavy chain usage in the response against HLA. Thus within a single, multi-specific serum we might see different distributions of IgG subclass for each specificity. Examination of sera containing multiple specificities allowed us to investigate whether the HLA specific IgG subclass profile is a property of the specificity or the serum. In each of 40 of the 51 sera analysed, multiple specificities were present which were considered not due to cross-reactions (because of temporal separation of the appearance of specificities and/or the absence of known cross-reactions). In seven of these all the specificities within each serum had the same subclass profile. In the remaining thirty-three sera, the clear majority, there was mixtures of different subclass profiles for the different specificities. Examples of sera showing different patterns of IgG subclass profiles for different specificities are shown in Table 6.6. In Table 6.6a, the serum has five specificities all with the same profile (all 4 subclasses present) even though there are two separate sources of immunization stimulating distinct specificities. This contrasts with the serum shown in Table 6.6b which has four specificities with differing profiles. The two specificities stimulated by graft mismatches, DR103 and DQ5, have the same subclass profile while the transfusion stimulated specificities, B57 and DR4, have their own peculiar subclass profiles (IgG1 and IgG2 only, respectively). These latter two specificities are likely to have been the result of distinct immunisations as they appeared about 5 months apart.

Table 6.6: Complex patterns of HLA-specific IgG subclasses vary within individuals and depend on route of sensitisation and specificity. Examples of antibodies in three sera from separate individuals (a-c). Values given are the ratio of MFI for specific reactions to the average of the negative control beads for each subclass to allow comparisons between subclasses (ie >5 is positive, as indicated by shaded boxes). The causes of each sensitisation is indicated by superscript (B-blood transfusion, T-failed kidney transplant, P-pregnancy).

	Specificity		IgG3	IgG1	IgG2	IgG4
a)						
	B57	P	7.5	112.7	24.1	11.9
	A2	B	7.6	102.9	42.3	16.6
	DR7	P	35.9	145.5	45.1	25.9
	DR9	P	14.1	73.9	21.9	20.3
	DR11	P	14.9	97.5	27.2	15.7
b)						
	B57	B	4.3	8.8	1.9	3.2
	DR103	T	2.5	180.2	317.1	9.3
	DQ5	T	2.7	152	340.7	132.6
	DR4	B	0.6	2	128.2	3.2
c)						
	B7	T	3.2	44.1	3.8	5.5
	A1	T	1.4	20.9	0.7	0.3
	DR4	T	1.1	16.7	0.6	2.3
	DQ7	T	9.1	112.8	6	62.5
	DR1	B	2.1	34	1.3	4.1

The serum described in Table 6.6c contains four specificities of differing subclass profile stimulated by a graft rejection. The DQ7-specific reactivity has all four subclasses present including a relatively high level of IgG4. In contrast the B7 specificity is composed of IgG1 and IgG4, while those of both A1 and DR4 are limited

to IgG1. A fifth specificity, DR1, in this serum appeared after a transfusion and is restricted to IgG1.

6.4 DISCUSSION

The complex nature of the HLA-specific humoral response has only really been possible to understand since the use of assays based on recombinant proteins. We now know that each expressed HLA allele carries multiple epitopes [159] which can all stimulate antibodies such that a positive crossmatch against a single mismatch could be due to one or many epitope-specific antibodies. Here we give a detailed description of a further layer of heterogeneity, that of IgG subclass distribution and the presence and relative level of each subclass as well as the mixture of subclasses. Our analysis suggests a major factor underlying this heterogeneity is the type of immunising event, but following such an event individuals can also stimulate distinct specificities with different subclass profiles. Failed transplant stimulated antibodies are characterised by both a wider range of subclasses and higher levels of each subclass. We routinely observe the emergence of an HLA DQ-specific response associated with chronic graft failure (unpublished observations) and the dramatic skew towards IgG4 of these antibodies implies a specific quality of the immune response. The combination of HLA DQ specificity and IgG4 therefore has the potential as a biomarker for chronic rejection.

It has been shown previously that IgG1 is the predominant subclass of HLA-specific IgG [158, 160] and in general this data agrees with this. Other protein alloantigens, such as those on red cells are also biased toward IgG2 [161]. If one looks at individual responses against different types of immunisation then a more complicated picture emerges. This is likely to be of direct relevance to transplantation, particularly

antibody incompatible transplantation, because of the different functional properties of each IgG subclass. IgG1 and IgG3 are the more effective at complement activation via complement factor C1q and Fcγ receptor binding. IgG1 only, IgG3 only, or both have indeed been shown to be the more pathogenic Rh-specific antibodies which cause haemolytic disease of the newborn[162]. Therefore the consequences of transplanting across a particular level of IgG1 or IgG3 may be different than the effect of an equivalent level of IgG2 or IgG4. Lobashevsky et al [157] reported antibody-mediated rejection (AMR)-free transplants associated with IgG2/IgG4 donor-specific antibody. On the other hand, Honger et al [158] were unable to show this in terms of AMR diagnosis but the high prevalence and higher levels of IgG1 compared with the other subclasses may have limited the statistical power of their analysis. However they did see a tendency towards increased AMR risk from the higher IgG1 levels and this is consistent with the findings of Griffiths at al [163]. Others have shown that differences in complement activation cannot always be explained by IgG subclass composition. While complement activation may not be the only pathogenic feature of HLA-specific antibodies this does illustrate that all the factors contributing to antibody heterogeneity are likely to be determinants of the pathogenicity of a recipient's HLA specific antibodies.

The presence or absence of each subclass indicates the degree of heavy chain class switching for each specificity. As class switching is T cell dependent, progression from IgG3 to IgG4 suggests enhanced T cell activation while a subclass limited response implies limited T cell activation. Thus, the tendency for multiple subclasses to be stimulated by graft rejection may be due to a greater degree of specific T cell activation compared with the response against a blood transfusion. The qualitative and quantitative characteristics of a graft rejection response may help to explain why re-transplantation against a previous graft sensitisation is considered to carry a particularly

high risk. This is in contrast to the impact of HLA specific antibodies stimulated by transfusion alone with a tendency towards lower levels and a bias towards IgG1 only. These can quickly decline after which transplantation against the same specificities does not carry high risk [164].

The description of subclass composition according to heavy chain gene order is useful as this clearly identifies class switching progression. In certain cases we observed "skipping" of subclasses. For example, in one individual we found the specificity HLA B7 with the IgG subclass profile "3-24" (MFI values 106;6;49;53, respectively). However, we know there is no general IgG1 defect in this patient as the same serum had IgG1 specific antibodies against three other HLAs, including B60 (MFI values 381;121;186;223) (which interestingly shares epitopes with B7). We saw six individuals with specificities having the profile "-1-4" but in all cases other specificities in the same individuals include IgG2 (an example of this is seen in Figure 6.6c). Thus such subclass skipping may be relatively common and seems to relate to processes peculiar to the response to an individual sensitising antigen rather than necessarily a general heavy chain gene defect.

A general weakness in HLA-specific antibody quantification and characterisation is the lack of reagent standards for each specificity. This is unlikely to be resolved because of the large number of possible epitope-defined specificities as discussed in chapter 5. Even two sera with the same specificity are likely to have different combinations and proportions of immunoglobulin classes and subclasses competing for the same binding sites. These problems are not peculiar to bead-based antibody characterisation, and cell based methods suffer the additional problem of lacking standardised levels of target protein. The use of an arbitrary cut-off value is also a problem but currently unavoidable because of the lack of an obvious step in MFI values between positivity and negativity. The position of the threshold is therefore the

issue and more studies will be needed before this could be standardised for each subclass. Honger et al [158] used greater than mean+3 standard deviations to define a positive. We used a cut-off based on the median values of control beads as the MFI values do not form a normal distribution and chose a relatively high cut-off value because of the greater clinical significance of higher antibody levels, although at the expense of increasing the chance of false negative assignments. Our subclass assay is less sensitive than the pan-IgG assay and this was also seen by Honger et al [158]; the greater avidity of the polyclonal secondary antibody is probably why this gives more sensitive detection. Despite these concerns, analysis of IgG subclasses is practical both for further quantitative and qualitative description of HLA specific antibodies and may help resolve why these are not always an obstacle to transplantation.

In conclusion, we have been able to demonstrate a remarkable degree of heterogeneity between HLA specific antibodies between and within individuals. Subclass composition is an important characteristic of HLA-specific antibodies as this will be one of the factors contributing to their direct pathogenicity, hence the need for a systematic description of this parameter. An understanding of pathogenicity of different combinations of antibody specificity and IgG subclass profiles may help interpret a positive pretransplant crossmatch. Even when two antibody specificities are stimulated by the same event, they may have distinct subclass profiles and could be treated differently with respect to exclusion of potential donors carrying the corresponding HLA. These latter two points will become increasingly relevant in the drive to overcome allo-antibody barriers in transplantation while improving outcomes. Finally, the progression towards less functionally effective subclasses as a cause or consequence of graft rejection may seem paradoxical, but, as class switching is T cell-dependent, differences in subclass use implies differences in T cell responses and this might have value in guiding both anti-T cell as well as anti-B cell therapies.

Chapter 7

HLA-INCOMPATIBLE TRANSPLANTATION: THE IMPORTANCE OF HLA-SPECIFIC IgG SUBCLASS ANTIBODIES

7.1 INTRODUCTION

Renal transplantation is limited by a shortage of organs, but does offer the best quality of life for those with end stage renal failure. Living donor transplantation (LDT) is more than an expedient deceased donor transplantation, and also offers a choice as to when transplantation is performed, in particular allowing transplantation before dialysis if required. The Department of Health has provided targets to increase the rate of transplantation in the UK and because the number of deceased donor organs continues to decline, a rise in the rate of live donor renal transplantation will be the most effective way to meet the targets. There are obstacles to transplantation and pre-formed donor-specific antibodies have always been one of the major barriers.

Patients previously exposed to foreign human leukocyte antigens (HLA) through transplant, blood transfusions or pregnancy often develop antibodies reactive to HLA. Such preformed antibodies are the cause of hyperacute rejection where the organ fails immediately following revascularization [21] because of complement-mediated damage to the kidney endothelium.

Currently 25% of patients waiting for deceased donor transplant are sensitised to HLA antigens. Also, in the UK alone, about 400 living donor renal transplants fail work-up each year because of donor-specific antibodies. Extracorporeal antibody removal to facilitate transplantation is being increasingly considered and a reasonable estimate of demand in the UK might be 50-100 cases per annum. This estimate is based on the use of plasmapheresis (PP) based protocols for living donor transplants[165]. If it were possible to reduce HLA antibody levels in all of those awaiting transplantation, there would be in excess of 1000 sensitised patients on the UK transplant list who would potentially benefit. Studies have shown that plasmapheresis and low-dose CMV-

Ig combined with traditional immunosuppression is effective in producing a specific and durable elimination of antibody to donor HLA[166].

Our transplant unit at the University Hospital Coventry & Warwickshire (UHCW) NHS Trust has been pioneering the work in antibody incompatible (both HLAi and ABOi) transplants in the UK and has done over 100 such transplants with preformed antibodies in the last ten years [90]. The protocol we use involves plasma exchange to reduce donor specific antibody (DSA) between 3-7 times before transplant based on the regular measurement of antibody levels.

Among the isotypes of HLA-specific immunoglobulin (Ig), IgG is considered to be the agent of humoral rejection with complement activation and Fc receptor (FcR) mediated activities being the effector mechanisms. HLA-specific IgM is rarely found in the absence of cognate IgG (D Briggs, unpublished observations) and at low titre is probably harmless [156], and IgA DSA may be protective [167]. IgG subclasses (1-4) exhibit functional differences such as complement activation and Fc receptor binding. IgG1 and IgG3 are the most effective at complement activation. Interestingly although IgG3 has the greater binding efficiency to complement component C1q, IgG1 is the subclass most effective at complement dependent cell lysis [168]. Neither IgG2 or IgG4 appear to bind to C1q although IgG2 can fix complement weakly, but IgG4 not at all.

In normal human serum IgG1 predominates (65%), followed by IgG2 (23%), IgG3 (8%) and IgG4 (4%). For specific antibodies these proportions can change as, for example, hyperimmunization can result in IgG4 restricted response [169]: IgG4 predominance in factor VIII inhibitors exemplifies this [170]. There are few studies investigating IgG subclass distribution in HLA-specific antibodies, and none considering individual specificities or looking at specific responses. From the limited number of relevant studies it seems that in HLA-specific sera (identified by lymphocyte binding) IgG1 predominates [160, 163], with recently published data suggesting that the

presence of non-complement fixing IgG2 and IgG4 donor specific antibody (DSA) do not correlate with antibody mediated rejection [157].

This chapter aims to explore the distribution and role of donor HLA specific IgG sublasses in the early pre and post-transplant period following HLAi renal transplantation using a modified luminex microbead method.

7.2 METHODS

7.2.1 Patients

Fifty one patients who received HLA antibody incompatible (HLAi) renal allografts between June 2003 and October 2008 at University Hospital Coventry and Warwickshire were included in the study. All recipients underwent flow cytometry (FC) and complement dependant cytotoxic (CDC) crossmatching prior to transplant. Initially, donor specific (DSA) and third party (TPA) antibody levels were monitored daily both pre- and post-transplant on the luminex platform using the Labscreen Single Antigen bead assay (One Lambda, Canoga Park, CA).

Prior to transplant patients were treated with five alternate day sessions of double filtration plasmapheresis (DFPP) with the aim of achieving FC crossmatch negative at the time of surgery. The number of DFPP sessions was adjusted up or down depending on the starting levels of DSA. In some cases the transplant was performed in the presence of CDC and or FC-positive crossmatch.

Immunosuppression consisted of mycophenolate mofetil 1000mg twice daily beginning 10 days prior to transplant with dose reduction if white cell count fell below $4.0x10^9$/l. Tacrolimus commenced 4 days prior to transplant, 0.15mg/kg/day with a target trough level of 10-15mg/l. Prednisolone, 20mg once daily, was started at the time

of surgery. Methylprednisolone, 500mg, was given during the transplant operation. Post-transplant, two doses of basiliximab were given on days 0 and 4.

Rejection was diagnosed by renal biopsy if renal function deteriorated, or clinically if rapid onset oliguria occurs with a concomittant rise in creatinine and DSA levels. The 51 patients included in the study were broadly divided into those who experienced an episode of acute rejection in the first 30 days post-transplant (n=26) and those who did not (n=25). Patient characteristics can be seen in Table 7.1.

Table 7.1: Characteristics of patient cohort. Patients are grouped into those who experienced an episode of rejection in the first 30 days post-transplant and those who did not.

	Rejectors	Non-rejectors
Number	26	25
Male / Female	10/16 (63%)	8/17 (47%)
Mean Age at Transplantation (years)	41	40.7
Mean Duration Treatment for ESRF (years)	11.8	11.2
Previous Transplants		
0	9/26 (34%)	10/25 (40%)
1	1/:26 (58%)	11/25 (44%)
2	0/26 (0%)	3/25 (12%)
3	2/26 (8%)	1/25 (4%)
Pre-treatment Crossmatch Status		
CDC+ve	3/26 (12%)	5/25 (20%)
FC+ve	13/26 (50%)	13/25 (52%)
Microbead +ve / FC-ve	10/26 (38%)	7/25 (28%)

7.2.2 Methods

Crossmatching (CDC and FC) were performed as previously described [91]. Donor specific and third party antibody levels were monitored using the Lasbcreen Singles bead assay (One Lambda, Canoga Park, CA). Single antigen bead specificities were selected for each patient to correspond with all DSA and transplant mismatch (TXMM) specificities, in addition selected TPA specific beads were included. All HLA-specific antibodies were determined by testing at least two separate samples from each patient, and all antibody specificities were assigned following careful consideration of reactivity patterns and comparison with patterns of known epitope specific reactivity.

Patient serum samples were chosen at specific time points in the transplant course; pre antibody removal therapy, 1 day pre-transplant, 1 day post-transplant, corresponding with peak post-transplant total IgG level, and 30 days post-transplant.

Antibody analysis was carried out in accordance with manufacturers instructions as previously described [91], goat anti-human IgG1-4 isotype specific monoclonal antibodies (Southern Biotech, Birmingham, AL) were used to determine levels of IgG subclass reactivity. Raw mean fluorescence intensity (MFI) values were used to denote positivity. Isotype specific monoclonal antibodies were validated by using luminex microbeads coated with IgG subclass specific human myeloma antibodies. Cross-reactivity between isotypes was observed at <5% (table 6.3). Positive threshold MFI levels for each HLA specific IgG subclass were as following: 120.6 (IgG1), 72.0 (IgG), 62.7 (IgG3) and 17.2 (IgG4). These values represent a value that is 5 times greater than the negative control bead incorporated into the assay.

7.2.3 Statistical Analysis

Each individual is associated with one of the groups – rejectors (R) or non-rejectors (NR) based on clinically identified outcome. Statistical Wilkinson rank test was performed in order to assess whether data from the different groups had similar median values. The null hypothesis of no difference was tested at the 5% level of significance, and this is presented by *p*-values.

Values of MFI for each IgG subclass (*i=1, 2, 3, 4*) were normalised by corresponding threshold levels for each subclass:

$$MFI_r = \frac{MFI_i}{\Delta MFI_i}$$

Index *r* means *ratio.*

Δ denotes subclass specific positive threshold value.

IgG subclasses were also grouped by their complement activating ability: strong ability to activate complement (IgG1 and IgG3), weak/no ability (IgG2 and IgG4).

7.3 RESULTS

7.3.1 Pre-transplant data

MFI_r values for IgG subclasses and for assigned groups of complement activating ability are presented in Table 7.2 and Figure 7.1. It can be seen (Table 7.2) that IgG1 is a more prevalent subclass of DSA-HLA in both rejector (89%) and non-rejector (72%) groups. In the non-rejector group, IgG1 (72%) is followed by IgG2 (60%), IgG3 (56%) and finally IgG4 (36%) in order of frequency. This is in accordance with previous findings [158] and with the normal sequence of abundance of these subclasses of immunoglobulin in serum. In the rejector group the order is different: the percentage of IgG4 and IgG2 is 65% and they both follow IgG1. IgG3 is least represented with 54%. This means that concentration of IgG4, normally least presented in serum, is higher in patients who experienced an episode of AMR (65% vs 36%).

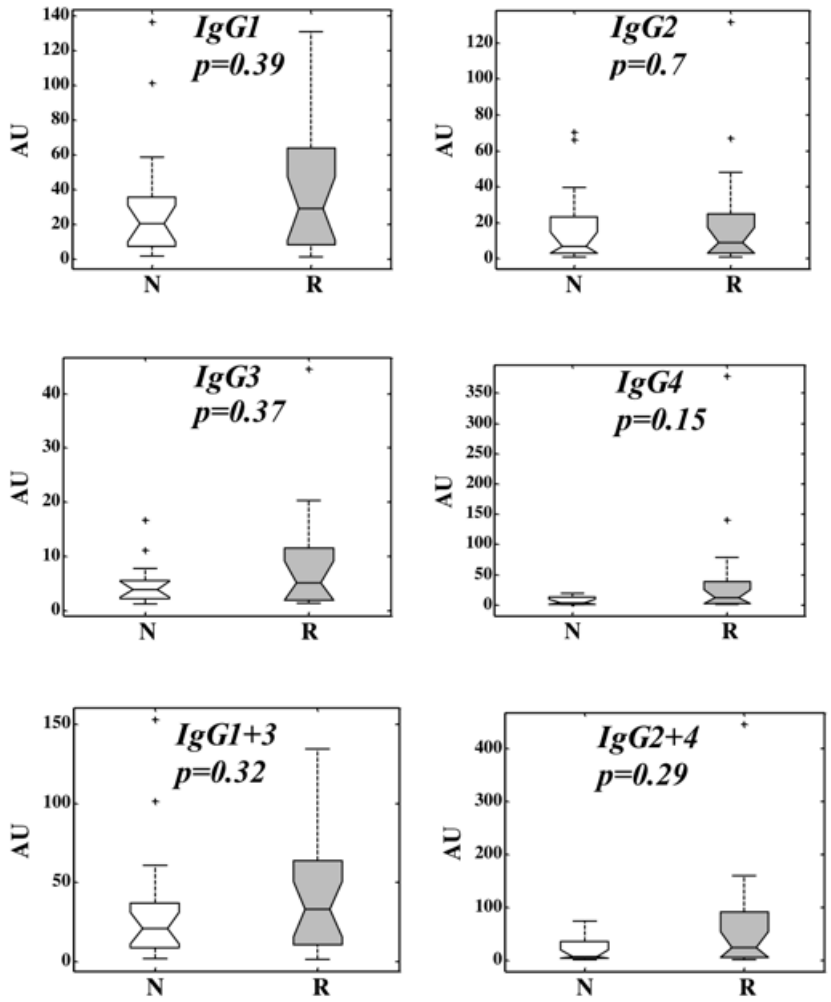


Figure 7.1: Box plots of pre-transplant MFI_r values for IgG subclasses. All are grouped by allograft rejection status: N –non-rejected cases, R – rejected cases.

[+] Denotes outlying values.

Another observation is that the range of antibody distribution shown in brackets in Table 7.2 for IgG4 and for joint IgG2 and IgG4 subclasses are extensive for the rejector group compared with non-rejector and with other subclasses, reaching relatively high maximal MFI values.

Table 7.2: Quantity and percentage (in brackets) of patients with presented IgG subclasses. Median MFI ratios and median ranges (in brackets) of IgG subclasses for pretransplant data are given representing all DSA's. All are grouped by clinical outcome.

	Rejectors (n=26	Non-rejectors (n=25)
IgG1	23 (89%) 29.3 (1.3-131)	18 (72%) 20.3 (1.6-136.4)
IgG2	17 (65.4%) 9.2 (1.2-131.6)	15 (60%) 6.8 (1.1-70.5)
IgG3	14 (54%) 5.1 (1.3-44.6)	14 (56%) 3.9 (1.2-16.6)
IgG4	17 (65%) 11.6 (1.4-378.2)	9 (36%) 3.3 (1.1-18.7)
IgG1+3	33 (1.3-134.4	20.9 (1.6-73.8)
IgG2+4	8.44 (0-445.1)	5.1 (0-73.8)

P-values presented in Table 7.2 and Figure 7.1 show that for samples taken before antibody removal therapy there is no statistically significant difference in MFI values for the two groups, either individually by IgG subclass or in two combined sets related to complement activating ability (IgG1+ IgG1 vs IgG2+IgG4): *p*-values calculated using Wilkinson rank test are all greater than *0.05*. This is also in correspondence with previously published results [14].

More detailed study of HLA class I and II DSA specificities was carried out for all IgG subclasses (Table 7.3) and did not show any statistically significant difference between R and NR groups either: *p*-values exceed 0.05. However, the calculated p-value is small (*p=0.08*) for IgG4 class II HLA, and additionally we observed a significant spread in MFI values and a much greater maximum value for class II IgG4 in

the rejector group than in non-rejector group. Another observation is that IgG4 is rarely seen in the non-rejector group: 3 out of 25 profiles (12%) compare with 46% (12/26) in the rejector group, indicating presence of this subclass in almost half of the profiles in the rejector group. This trend towards IgG4 is explored further.

Table 7.3. Quantity and percentage (in brackets) of patients with presented IgG subclasses. Median MFI ratios and median ranges (in brackets) of IgG subclasses for pretransplant data. All are grouped by clinical outcome.

	HLA Class I		HLA Class II	
	Rejectors (n=26	Non-rejectors (n=25)	Rejectors (n=26	Non-rejectors (n=25)
IgG1	17 (65.4%) 14.6 (1.6-131)	11 (44%) 40 (1.1-101.2)	17 (65.4%) 22.0 (1.3-77.5)	10 (40%) 20.3 (2.0-90.2)
IgG2	12 (46.2%) 5.3 (1.4-8.9)	10 (40%) 7.5 (1.1-66.2)	11 (42.3%) 8.0 (1.2-131.6)	8 (32%) 4.6 (1.1-42.9)
IgG3	9 (34.6%) 1.8 (1.3-15.7)	9 (36%) 4.8 (1.2-11.1)	10 (38.5%) 14.4 (1.4-378.2)	6 (24%) 2.9 (1.2-7.8)
IgG4	11 (42.3%) 2.3 (1.1-140.4)	10 (40%) 7.5 (1.1-66.8)	12 (46.2%) 144 (1.4-378.2)	3 (12%) 1.6 (1.1-1.9)
IgG1+3	16.2 (1.6-134.4)	11.1 (1.6-101.2)	26.6 (1.3-95.5)	22.3 (2-98)
IgG2+4	7.0 (1.1-143)	19.4 (2.5-72.3)	18.2 (1.4-445.1)	6.0 (1.1-44.5)

7.3.2 Post-transplant data.

There were two time points of interest in the post-transplant antibody course: that which corresponded to the peak level of pan-IgG DSA (days 8-11) and antibody levels on the 30th day after transplantation where in most cases post-transplant IgG responses had normalised back down to baseline levels.

Peak level distributions for all 51 patients are presented in Figure 7.2A. If there is a significant difference in median values between the two groups the values are marked by an asterix. The results are qualitatively similar to the pre-transplant data for IgG2, IgG3, and IgG4 subclasses (Figure 7.2A) However it can be seen that there is a significant increase in peak values in the rejector group of donor-specific IgG1 subclass with *p*-value equal to *0.01*. Also, the proportion of the total IgG response associated with the complement fixing subclasses IgG1 and IgG3 is greatly increased in the R group ($p=0.007$) in comparison with the pre-transplant value ($p=0.32$). More detailed study of DSA-HLA specificities of class I and II carried out for all IgG subclasses showed that IgG4 class II (Figure 7.2B) associates with R group with the *p*-value further dropping to *0.04* compared to pre-transplant data (Table 7.3, $p=0.08$). The form of the distribution also differs from the others in that the spread of IgG4 MFI values in the rejector group is significantly higher and the maximum MFI value is higher than for all other groups/subclasses.

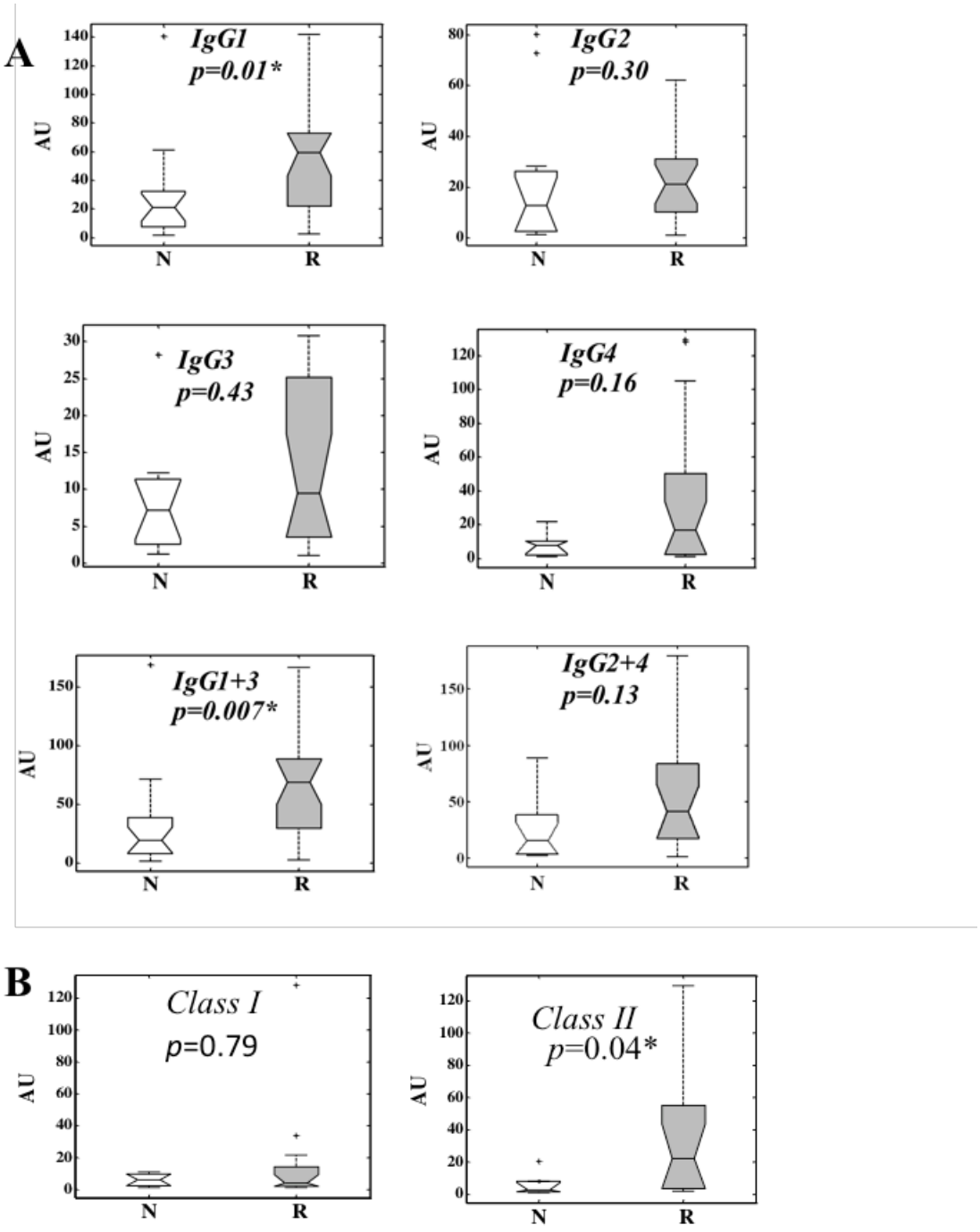


Figure 7.2: DSA subclass distribution at the peak post-transplant timepoint. A) Box plots of peak post-transplant MFI values for IgG subclasses. All are grouped by allograft rejection status: N –non-rejected cases, R – rejected cases. B) Box plots of peak post-transplant MFI values for IgG4 class I and II HLA grouped by allograft rejection status: N –non-rejected cases, R – rejected cases. $^{+}$ Denotes outlying values.

Next, we compared the amount of IgG subclasses presented in rejector and non-rejector groups for the 30th day after transplantation. This is shown in figure 7.3.

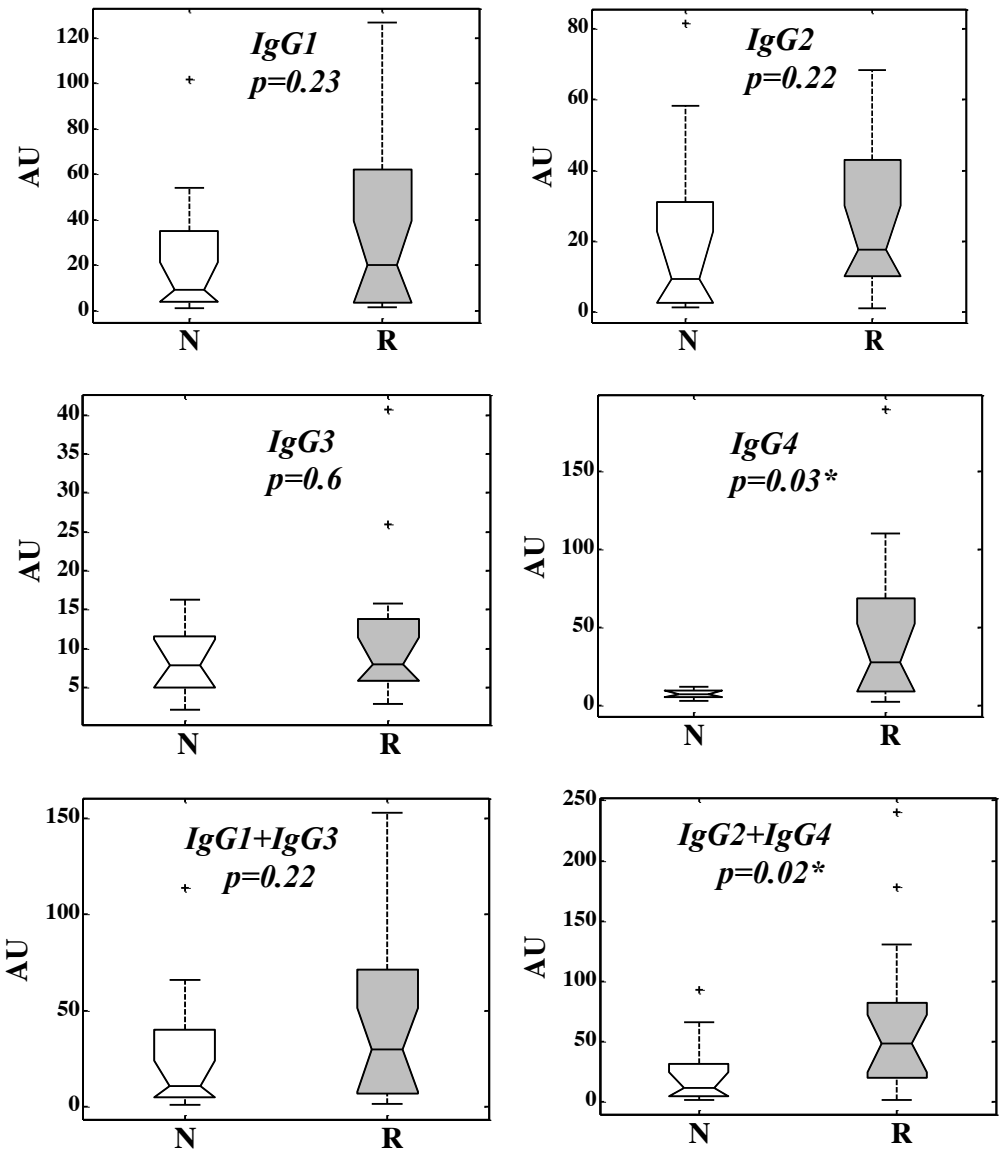


Figure 7.3: Box plots of 30th day post-transplant MFI values for IgG1, IgG2, IgG3, IgG4, IgG1+IgG3 and IgG2+IgG4. All are grouped by allograft rejection status: N –non-rejected cases, R – rejected cases. + Denotes outlying values.

There was no difference between rejector and non-rejector groups for presence of any individual subclass. However, the rejection group demonstrated a significant increase in donor specific IgG4 with *p*-value *0.03* (Figure 7.3). In addition, the proportion of the total IgG response associated with weak/no complement activating ability subclasses IgG2 and IgG4 increased in the rejector group (*p=0.02*). Concentration of IgG4 in the rejector group is twofold greater than that in the non-rejector group.

7.3.4 IgG4 Further Analysis

In order to fully investigate the observed association with AMR of increased levels of IgG4, a further cohort of 35 patients (19 rejector, 16 non-rejector) were tested at pre-transplant, peak post-transplant IgG4, and 30 days post-transplant time-points, specifically for presence and levels of IgG4 specific DSA. These additional patients are an extension of the original cohort and represent the next group of HLA incompatible transplant patients from our cohort.

Figure 7.4A shows the Wilcoxon rank test data from both cohorts individually and combined. The increased sample size allows us to identify a significant increase in class II DSA specific IgG4 both pre-transplant and also at the peak post-transplant IgG time-point. There is also a significant increase in 30 day post-transplant IgG4 when HLA class I and II are analysed together. Figure 7.4B shows the HLA class II donor-specific IgG4 for the large sample group at each time-point. The overall analysis of median reactivity for each subclass shows that the trend for IgG4 is atypical when compared to the other subclasses (Figure 7.4C), with IgG4 levels continuing to rise

even at the 30th day post-transplant at which point all other subclasses have reduced considerably from peak post-transplant levels.

A

First cohort	Pre-TX	Peak-TX	30th day
Class I+II	0.15	0.16	**0.03**
Class I	0.77	0.79	0.71
Class II	0.08	**0.04**	0.36

First + second cohorts	Pre-TX	Peak-TX	30th day
Class I+II	**0.01**	0.09	**0.026**
Class I	0.81	0.63	0.78
Class II	**0.02**	**0.03**	0.21

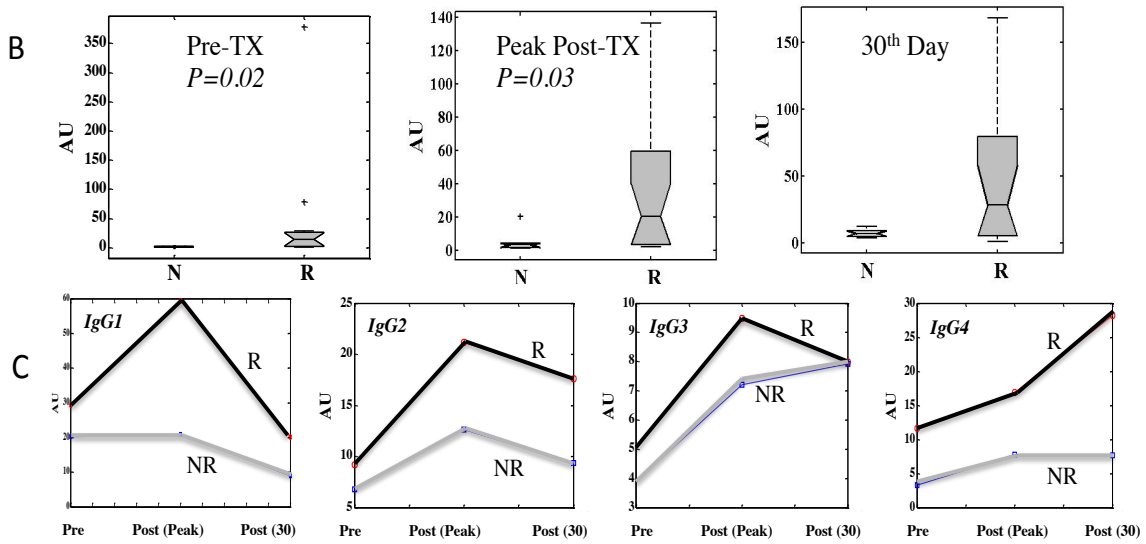


Figure 7.4: Kinetics of HLA-specific IgG subclasses. A) Differences in donor-specific IgG4 between rejector and non-rejector groups. Significant differences between groups are highlighted in bold. B) Box plots to show differences in IgG4 level between rejector and non-rejector groups. C) Median MFI value calculated over the group of rejectors (black line) and non-rejectors (grey line).

7.4 DISCUSSION

A recent study described the use of a modified single antigen bead assay to demonstrate that analysis of subclass specific DSA prior to transplantation did not provide any substantial additional value beyond the standard pan-IgG assay in the risk stratification of potential transplants [158]. This data set sought to confirm this observation, but also to investigate the nature of subclass specific responses post-transplantation. Regarding pre-transplant risk assignment it was also initially observed that no individual subclass or combination of subclasses based on putative complement fixing ability was predictive of AMR in the early post-transplant period. However, a *p* value of 0.08 associated with the presence of IgG4 DSA directed against HLA-class II was observed. This was confirmed by the testing of an additional cohort of 35 patients, which strengthened this association (p=0.02). Due to the unusually high concentration and extensive range of MFI values of IgG4 subclass, and particularly HLA class II specific DSA, the presence of IgG4 specific for donor HLA class II in serum before antibody removal therapy and before transplantation could be an indicator of high rejection risk.

Analysis of subclass profile at the time of peak post-transplant pan-IgG donor reactivity showed two significant observations. Firstly that the levels of IgG1 specific DSA had risen considerably from pre-transplant levels for the rejector group (p=0.01), and secondly that the proportion of the DSA response that could be attributed to complement fixing subclasses (IgG1 and IgG3) had skewed heavily towards this strong complement fixing profile (p=0.007). Interestingly, IgG3 in isolation was not significantly increased in those who experienced AMR. This is in contrast to recent studies suggesting an association between IgG3 and rejection[171, 172]. These associations may be due to the fact that in these studies it appears that the IgG3 DSA

detected are *de novo* and implicated more in the chronic rejection setting. In our cohort we were able to identify these antibodies prior to transplant and in many cases we can assign them to a specific historical sensitising event. This implied memory response would lead us to suspect that class switching has long since progressed past IgG3, and given that the half-life of IgG3 is only around 9 days we would not have expected to see an over-representation of IgG3 in our cohort. Additionally the levels of IgG4 DSA were also increased at this point, although when class I and II DSA were combined this did not reach statistical significance in the original cohort ($p=0.15$). However, separate analysis of HLA class II specific DSA shows a significant shift towards IgG4 in the rejector group ($p=0.04$), and this observation was even stronger following the testing of an additional 35 patients ($p=0.03$). This observation suggests that antigen dependant T-cell driven affinity maturation processes are active as a significant component of the DSA is shifting towards production of IgG4.

Analysis of the 30 day post-transplant sample also shows the increased contribution of IgG4 DSA in the rejector group. The analysis of median reactivity of all subclasses shows that the IgG4 response in the rejector cohort in particular continues to rise at least until 30 days post-transplant. All other subclasses show reduction from peak post-transplant levels by this point. This suggests that the process of affinity maturation is ongoing and that increasing numbers of memory B cells are switching toward IgG4 production then maturing into subclass committed plasma cells. We believe this observation to be novel, and has been hitherto undetectable using the pan-IgG assay as the relative contribution to the overall DSA response by IgG4 would always be miniscule in comparison to other subclasses, in particular IgG1. The fact that this response is dominated by reactivity to HLA class II antigens is interesting and potentially sheds light on the way in which the humoral response contributes to chronic rejection. In our laboratory, and many others, it is a common observation that chronic

rejection episodes are often characterised by donor specific antibodies directed against HLA class II, in particular HLA-DQ. In this study the post-transplant subclass specific response is only analysed up to the 30 day post-transplant sample, a further analysis of patient samples following the transplant course for much longer is indicated to determine the extent and duration of the progressive class switching and affinity maturation. The association of increased IgG1 in the rejector group at peak IgG time-point was lost at 30 days post-transplant as IgG1 levels fall back towards baseline levels, it is the dynamics of the IgG1 response that typifies the overall pan-IgG profile we observe routinely in these cases[91].

The subclass specific microbead assay itself is robust, with each subclass specific monoclonal antibody validated using a panel of carefully prepared subclass specific human myeloma immunoglobulins. Acceptable levels of cross-reactivity were observed between all subclasses, although as reported in other studies the greatest degree of cross-reactivity was seen with the IgG2 subclass specific monoclonal antibody. Similar to other groups we noticed a decreased sensitivity of the subclass specific assay when compared to the pan-IgG standard assay. It should also be noted that in many cases multiple subclass specific antibodies recognising the same epitope(s) would be present in the same sera. In theory this may lead to competitive inhibition of binding to single antigen beads and may mean that certain subclasses with reduced affinity may have reduced binding in the assay. We have balanced this reduced sensitivity by altering our positive cut-off threshold accordingly, with a cut-off value of greater than 5 times the internal negative control bead deemed positive. This is a stringent way of determining positivity and as such provides a greater risk of assigning weakly positive samples as negative. Therefore it may be that many of the observations here have underestimated the number of positive samples and that in turn all positive associations carry greater weight for this. The use of a ratio of reactivity (number of

times greater than the negative control bead), allows us to use a ranking analysis for most of the data, which has the added advantage of eliminating the need to define a positive/negative reaction cut-off.

In conclusion, this data strongly suggests that there is merit in determining the donor-reactive IgG subclass profile prior to transplantation, as increased presence of donor-reactive HLA class II specific IgG4 appears to be predictive of early AMR. In addition, the careful monitoring of IgG subclasses in the early post-transplant period is highly informative, with a skewing of IgG subclasses towards IgG1 and the strongly complement fixing IgG1+IgG3 combination strongly associated with AMR. The early identification of this shift in subclass composition may indicate clinical intervention and reduce the impact of AMR in these high-risk transplants.

Chapter 8

SELECTIVE ANTIBODY DEPLETION USING HLA COLUMNS

8.1 INTRODUCTION

HLA-specific antibodies have become a dominant problem in solid organ transplantation. Such antibodies cause early loss of renal allografts if present at the time of transplantation, and approaches to transplanting across anti-HLA antibodies are only partially successful [99, 105, 129]. HLA-specific antibodies cause long term graft failure in kidney and other solid organ transplants [24, 173]. Despite the realisation that these antibodies are of such importance, our ability to effectively suppress synthesise and remove these antibodies from the patient blood are limited. Clinical outcomes would be markedly improved if we were able to define antibody specificities and selectively control their synthesis, and also selectively remove large amounts of HLA antibodies from patients.

The ability to synthesise and purify HLA proteins has recently transformed the ability to measure HLA antibody levels in patients' serum, as the proteins have been coupled to microbeads and have allowed rapid measurement of multiple antibody specificities from small amounts of serum. Reproducible solid phase assays have not completely replaced cellular crossmatch techniques, but are now a mainstay of HLA antibody screening and monitoring [91, 174].

One new application would be better to define the specificities of HLA antibodies. These are not related to HLA specificities *per se*, but to the epitopes and more specifically eplets on HLA proteins. An HLA molecule may be capable of stimulating the production of more than one anti-HLA antibody specificity, and an antibody directed against an epitope on one HLA protein may react with the same epitope on many other HLA proteins [81, 146]. Many HLA epitopes have been defined [80, 147] but it is not yet possible to use this understanding to define a patients' HLA

antibody profile by epitope. It is still necessary to list HLA specificities, which is cumbersome, imprecise, and may prohibit transplantation unnecessarily.

Methods currently available for the removal of HLA-specific antibodies from patients are sufficient to allow the successful transplantation of those with moderate levels of donor HLA specific antibodies (DSA)[90, 91, 107, 116, 129]. A commonly used method of antibody depletion is plasma exchange, where patient blood is passed through a plasma filter or is simply centrifuged. The plasma component, which contains the antibodies, is removed and discarded. However, along with the antibodies the plasma component also contains albumin and other clotting factors and these have to be replaced with fresh donor plasma. Depending on the size of the patient, only 3-4 litres of plasma can be treated per session. Double-filtration plasmapheresis (DFPP) is a more refined approach whereby plasma is separated as for plasma exchange but is then passed through a second filter which operates via size exclusion. This filter traps larger molecules and consequently smaller plasma proteins which need to be replaced in standard plasma exchange such as albumin and lower molecular weight clotting factors are allowed to pass back into the patient. This approach is much better tolerated by the patient and typically DFPP treatment volumes can be 6-8 litres per session, usually only limited by fluid shift out of the vascular compartment [108]. A slightly more selective approach can be taken using protein A immunoadsorption. Once again plasma is separated as for DFPP and is then passed through a column containing immobilised protein A. Protein A binds human IgG, with the exception of the potent complement fixing IgG3 isotype. It is therefore a highly effective method for removing antibody prior to transplantation. Following passage through a protein A column only the antibody molecules are removed leaving all other plasma proteins unaffected which makes this a very well tolerated treatment modality, with treatment volumes of up to 40

litres in a single session possible [110]. The major disadvantage of protein A immunoadsorption is its high cost and additional removal of essential, protective IgG

However, these methods cannot remove sufficient antibody pre-transplant to allow safe transplantation of those with high levels of donor specific antibodies (DSA), and post-transplant the non-specific nature of current methodologies such as DFPP or Protein A columns means that all antibodies are depleted, leading to a significant infection risk, especially when antibody removal is performed in patients on intense immunosuppression. The ability to remove selectively large amounts of HLA-specific antibody before and after HLA antibody incompatible (HLAi) transplantation would make this treatment approach much safer and enable many more renal transplants to take place, and might also be useful for other organ transplants. Selective antibody removal has been possible for some time in the ABO incompatible transplant setting following the introduction of Glycosorb columns, where the only component removed is the ABO blood group specific antibody that is bound to the column, everything else in the patient plasma passes back into the patient. This makes this procedure very well tolerated with up to 10 litres of plasma treated in a single session being reported [111].

In this study we set out to develop and test a method to selectively remove HLA specific antibody from human blood.

8.2 METHODS

8.2.1 Patients

Serum samples were taken from our archive of 100 HLAi transplant patients. The study was approved by the West Midlands Research Ethics committee and written informed consent was obtained. The HLA specific antibody profiles of these patients have been elucidated to the highest available resolution by single antigen bead assay. Patients were chosen on the basis of having high reactivity (>5000 mean fluorescence intensity (MFI)) for the specific HLA antibody studies, as measured by luminex single antigen bead analysis.

For clinical scale column analysis larger volumes of plasma effluent, a by-product of DFPP were used for analysis. Briefly, 500-600ml of patient specific plasma effluent was centrifuged at 4000g for 45 minutes and supernatant filtered through 0.45μM. The filtered plasma effluent was then diluted 1:4 with HLA-specific antibody negative fresh frozen plasma (FFP) to a final volume of 2 litres. A specific anti-HLA reactivity in the range 5-10,000 was determined by luminex single antigen bead analysis.

8.2.2 Soluble HLA-A*02:01 / B*07:02 Protein Production

To produce secreted HLA-A2, α-chain cDNA of HLA-A*02:01 or HLA-B*07:02 was modified at the 3' end by PCR mutagenesis to delete codons encoding the transmembrane and cytoplasmic domains and the VLDLr purification epitope added. The resulting construct was cloned into the mammalian expression vector pcDNA3.1(-) that contained a geneticin resistance cassette. Class I HLA deficient cell line 721.221

was transfected with the expression construct and drug resistant clones were selected in growth media containing G418 (0.8mg/ml). sHLA production was measured using a capture ELISA where W6/32 was the capture antibody and anti-β2m was the detecting antibody. sHLA producing clones were sub-cloned into 96 well plates by limiting dilution and high producing sub-clones were expanded and seeded in AccuSyst-Maximizer hollow fiber bioreactor (Biovest International). Approximately 500 mg of sHLA-A2 was harvested from the bioreactor. sHLA-A2 containing supernatant was loaded on a W6/32 immunoafffinity column and washed with 40 column volumes of 20mM phosphate buffer pH 7.4. sHLA-A2 molecules were eluted from the affinity column with 50mM DEA at pH 11.3, neutralized with 1M TRIS pH 7.0, and buffer exchanged and stored at 1 mg/ml in sterile PBS.

8.2.3 Class I Single Antigen Bead Assay

HLA class I specific antibodies were analysed using a recombinant single antigen microbead assay manufactured by One Lambda Inc. (Canoga Park, CA) and analysed on the Luminex Xmap 200 platform (Qiagen, UK). Antibody binding was measured as raw fluorescence to avoid differences in background binding seen with different sera which disproportionately influences relative fluorescence, a particular problem associated with DFPP therapy. All assays were performed using serum/bead ratios in accordance with the manufacturer's instructions. Raw MFI values were used to determine anti-HLA antibody specificity.

8.2.4 HLA Protein Mini-column Coupling Protocol

To prepare a 200µg HLA protein column, 200mg freeze-dried cyanogen bromide (CNBr) activated Sepharose-4 Fast Flow matrix (GE Healthcare, NJ,USA) was swollen and activated using 2ml 1mM HCl pH3.0 and chilled on ice for 30 minutes. The swollen matrix was then centrifuged at 2000g for 10 minutes and supernatant was discarded. Matrix was then resuspended in 2ml suspension buffer (50mM HEPES, pH7.8, 100mM NaCl), then re-centrifuged at 2000g for 10 minutes. Matrix was then resuspended in 500µl suspension buffer to give a final matrix concentration of approximately 2mg/ml.

The starting concentration of HLA protein was determined using OD_{280} absorbance measurement. 200mg of HLA protein was added to 100µl matrix and incubated for 2 hours at 4°C. The matrix was centrifuged at 2000g for 5 minutes and the OD_{280} absorbance of the supernatant was determined to confirm coupling efficiency greater than 80%. Non-reacted matrix residues were then dectivated by the addition of 1ml 1M ethanolamine and incubation at 4°C overnight. Supernatant was then removed and the matrix resuspended in 1ml PBS containing 0.05% sodium azide (NaN_3) pH7.4. The protein coupled matrix was then packed into a 2ml affinity chromatography column. A negative control column was prepared in parallel using bovine serum albumin (BSA) as an alternative to HLA protein.

8.2.5 Clinical Scale HLA Protein Column Coupling Protocol

sHLA-A2 was covalently linked to Sepharose 4 Fast Flow using NHS activated, amine-based chemistry (GE Life Sciences). For the coupling reaction 1mg of sHLA was added per 1ml of swollen matrix in alkaline buffer (0.1N $NaHCO_3$ pH8.3). After 1 hour

of coupling, the reaction was stopped. Uncoupled NHS groups were blocked with 0.1M TRIS pH8.3 for 2 hours. After blocking, the matrix was washed and stored in PBS, 0.02% NaN_3. For all matrix the used in this study, the coupling efficiency was ≥90%.

8.2.6 Antibody Removal using HLA Protein Columns

Mini-columns were tested for antibody binding capacity by treating repeated cycles of 1mg HLA-specific monoclonal antibodies W6/32 and Beta 2 microglobulin (B_2M). 1ml of patient serum was applied to the HLA protein column containing A2 antibody specificity and allowed to run through by gravity. The negative control BSA column was run in parallel. The post-column serum fractions were then re-tested with class I single antigen beads. To analyse the characteristics of antibody eluted from the columns 5ml 100mM glycine pH11 was added to the column and the eluate was immediately neutralised in 1M Tris-HCl pH7. Eluted fractions were dialysed into PBS pH7.4 and analysed using single antigen bead assay.

Clinical scale HLA columns were tested for antibody binding capacity by treating with 25mg of class I HLA-specific monoclonal antibodies W6/32 and B_2M. Columns were set up in a simple circuit which first allowed 1 litre of NaN_3 free PBS to be pumped through the column. Pressure monitors were set up proximally and distally to the HLA column in order to monitor the fluid pressure through the column and potentially identify any build up in pressure indicative of column blockage. The circuit was then switched to allow the diluted patient plasma to flow through the HLA column at a flow rate of 50ml/min used clinically with Glycosorb. 1ml aliquots of plasma were taken after flowing through the column for further analysis at specified timepoints. After the circuit was switched back to NaN_3 free PBS to fully flush the column clear of any unbound protein remaining from the patient plasma run. During the antibody

elution phase, 1 litre of 100mM glycine pH11.0 was passed through the column and 25ml aliquots collected, which were immediately neutralised in 5ml 1M Tris pH7.0.

8.3 RESULTS

8.3.1 Optimisation of Column Elution Conditions.

The soluble HLA (sHLA) proteins used to produce the mini-columns are sensitive to acidic pH's below pH6.5. Therefore traditional acid elution would irreversibly damage the integrity of the sHLA. In order to re-use the HLA columns a suitable elution buffer which utilises an alkaline pH needed to be identified. A stock solution of 1M glycine was prepared, then a pH range of 8-12 was prepared in 100mM glycine. W6/32 was passed through a 1ml HLA-A2 column then eluted with each buffer in turn with rising pH. The combined elution profiles for each buffer is shown in figure 8.1.

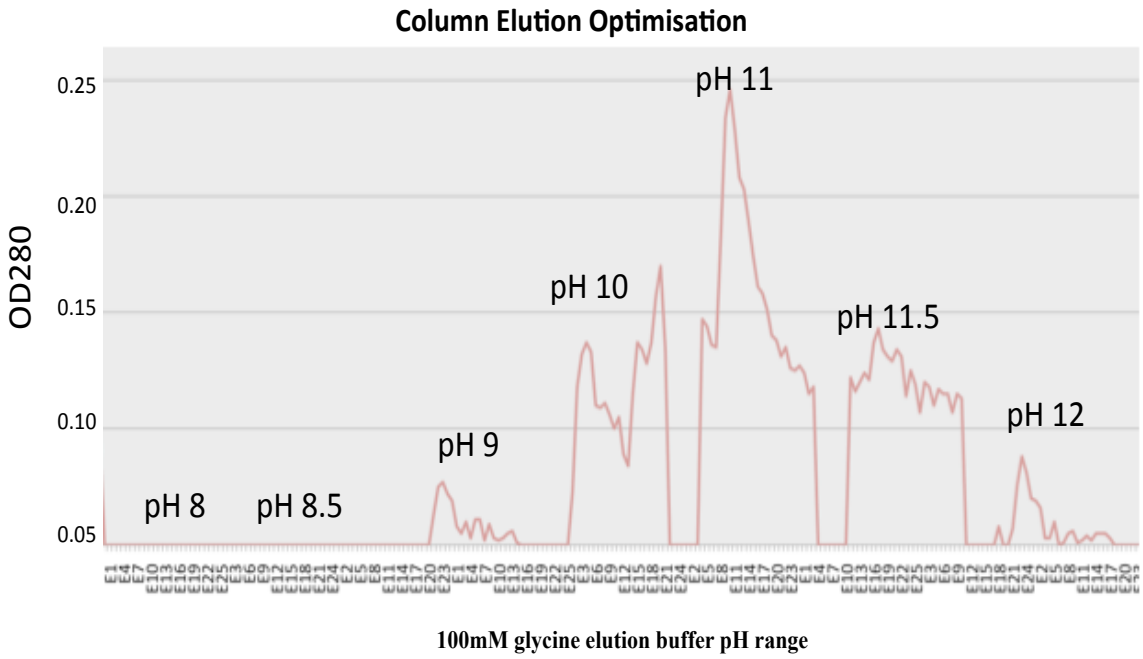


Figure 8.1: Optimisation of elution conditions. Solutions of 100mM glycine were set up at a range of pH values. pH11 was clearly shown to be most effective at HLA-specific antibody isolation

Peak elution of W6/32 monoclonal antibody was seen at a pH of 11, with the total amount of eluted monoclonal as determined by OD280 absorbance of 250µl. Effective elution is observed between pH10 and 11.5. Long-term storage of eluted antibody fractions required a neutralisation of the pH with the addition of 1M Tris pH7.

8.3.2 Mini-column Validation

Having determined the optimum conditions for antibody elution, HLA-A2 and B7 mini-columns were assessed for efficacy and reproducibility of elution conditions by running through the HLA-Class I specific monoclonal antibodies W6/32 and B2M at a concentration of 1mg/ml. Column capacity was determined by measuring the OD280

absorbance of the eluted fractions. Column performance was analysed for a minimum of 3 cycles of each monoclonal. Performance trend for each column is given in figure 8.2.

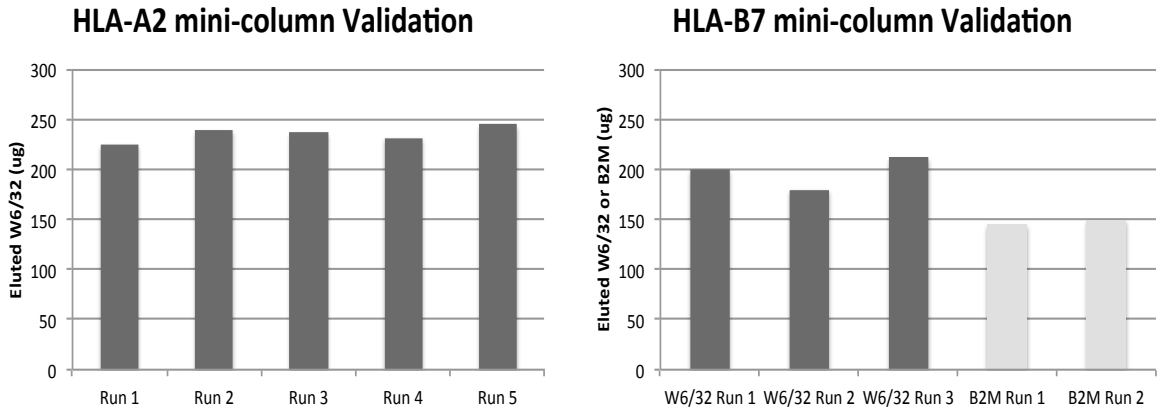


Figure 8.2: HLA-A2 and HLA-B7 mini-column validation. Capacity of 1ml mini-column to isolate W6/32 or B2M monoclonal antibody was maintained following multiple absorption / elution cycles.

Column efficiency was consistent over repeated cycles of antibody binding / column regeneration with a binding capacity in the region of 200-250µl of HLA specific antibody which was consistent after multiple elution cycles. Capacity of the 1ml HLA column is therefore unaffected by the extreme alkaline pH required to elute antibody from the HLA column.

8.3.3 Reduction capacity of 1ml mini-columns

Serum samples from patients who were included in our HLA incompatible (HLAi) renal transplant programme were selected if antibodies directed against HLA-A2, B7 or both were consistently detected by luminex microbead analysis. Pre-column

HLA antibody reactivity (as shown by the mean fluorescence intensity [MFI] value) was determined by microbead analysis. Sera (typically 5ml) was then passed through the HLA-A2 or HLA-B7 mini-column and then re-analysed for HLA antibody reactivity by microbead analysis. Figure 8.3 shows the reduction in MFI post-column in six patients positive for anti-A2 and six HLA-B7 antibody positive patients.

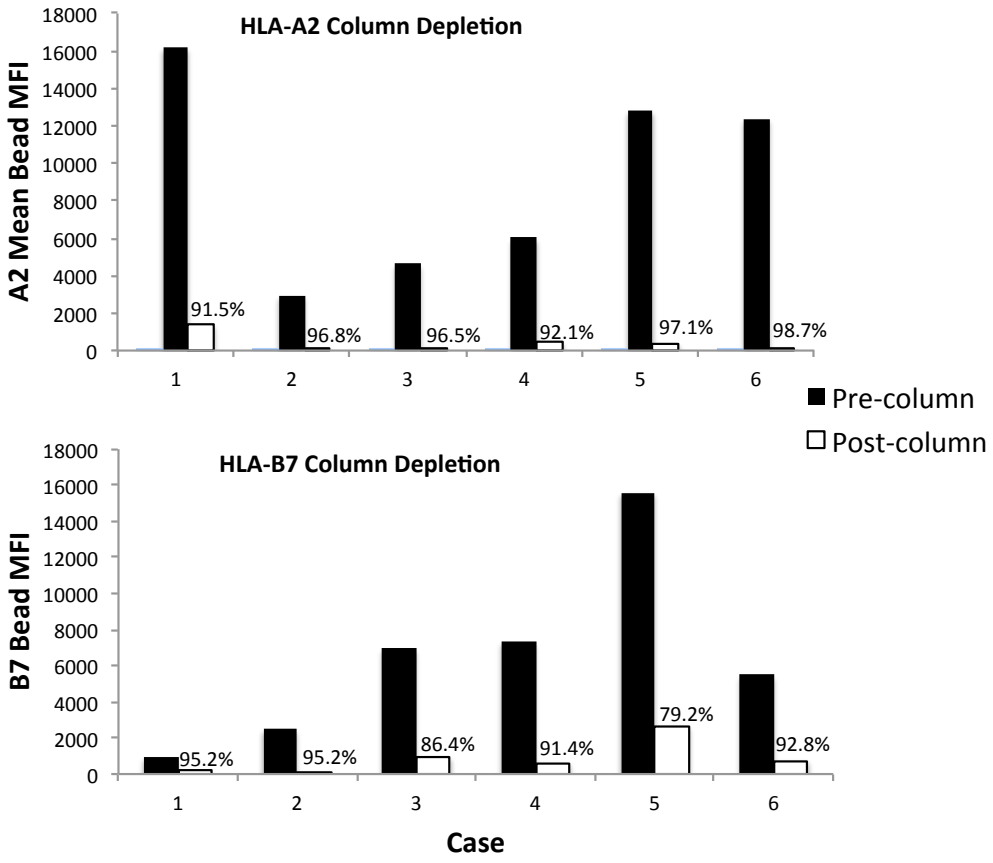


Figure 8.3: Reduction in MFI reactivity following antibody depletion using HLA-A2 and HLA-B7 mini-columns. In all cases mini-column depletion resulted in a significant depletion of DSA reactivity measured by microbead assay.

Figure 8.3 clearly indicates that in all cases depletion of HLA-A2 specific antibody based upon MFI readout was highly effective with a mean percentage antibody

removal of 95.45% (range: 91.5-98.71%). HLA-B7 specific antibody removal was also highly effective with a mean antibody reduction across all six cases of 93.04% (range: 79.22-95.24%).

8.3.4 Specificity of Antibody Eluted from the HLA-column

The mini-column had shown excellent capacity to deplete HLA-specific antibody, with 1ml columns depleting up to 5ml patient sera. The antibody removed by the column was then eluted from the column for further analysis. Figure 8.4 shows the elution profiles of three patients whose sera was passed through a 1ml HLA-A2 mini-column and three whose sera was passed through a 1ml HLA-B7 mini-column. Luminex microbead analysis was performed on fractions taken during the load phase (when patient sera was passed through the column), the wash phase (rinsing with 4ml PBS prior to elution), and the elution phase (antibodies released from the column by alkaline elution).

The data in Figure 8.4 clearly indicates that the mini-columns elute HLA-specific antibody in an epitope specific manner. Cases i and iii show that 3rd party antibody (i.e antibody that does not recognise HLA-A2 or B7 specific epitopes) passes through the mini-column in the load and wash phases. HLA-A2/B7 specific antibody is absorbed onto the column matrix, as supported by the excellent reduction in MFI values detailed in figure 8.3. Under appropriate alkaline elution conditions this HLA-specific antibody is released from the column and detected at a range of concentrations by microbead analysis. The antibody that is eluted from the column shows not only HLA-A2 or B7 specific reactivity but also excellent reactivity against a range of antigens which share common epitopes with HLA-A2.

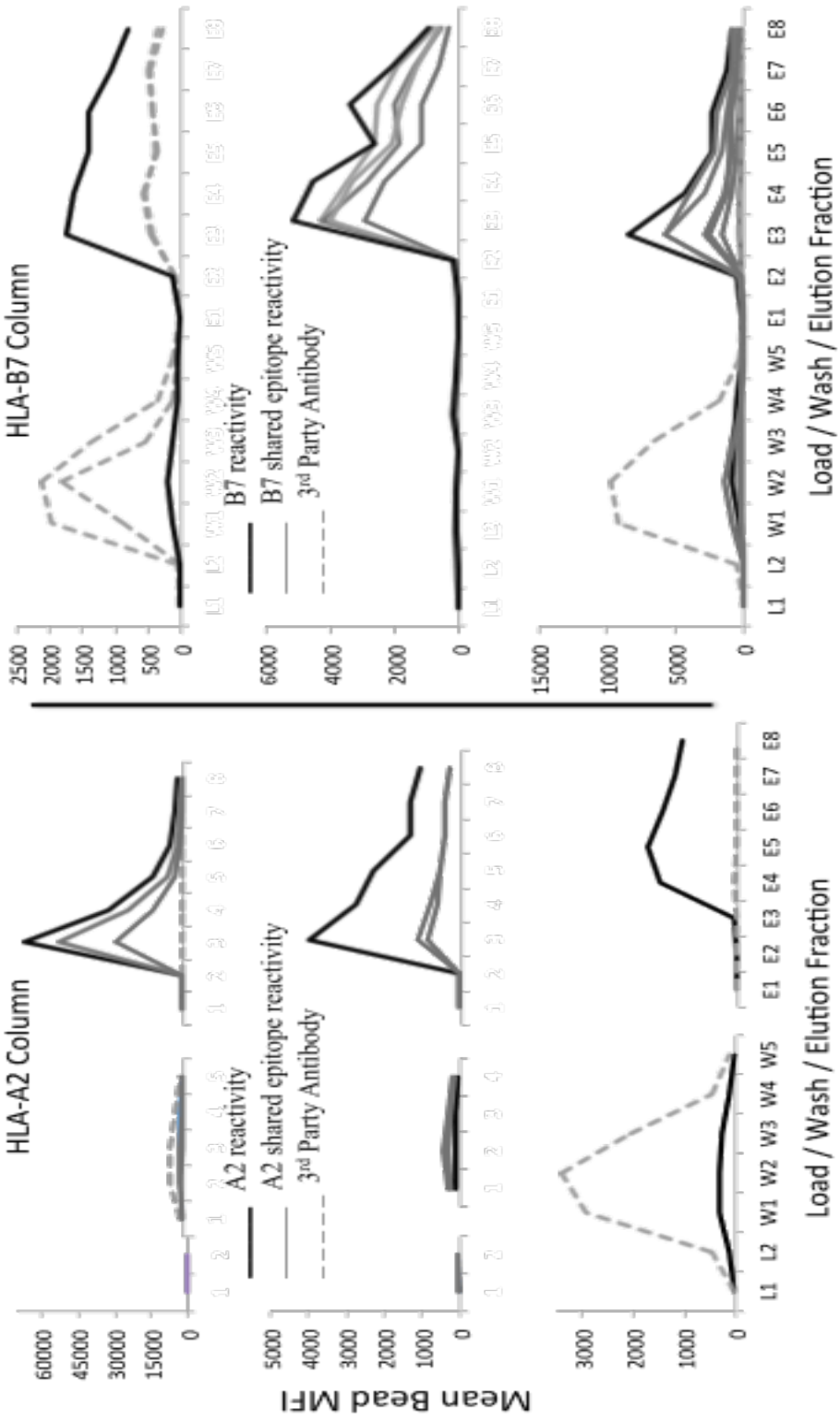


Figure 8.4: Epitope specific absorption using 1ml HLA Columns. Selected patients were passed though HLA-A2 of HLA-B7 1ml columns. Antibody was absorbed and eluted in an epitope specific manner

8.3.5 Effect of Mini-Column Depletion on Crossmatch Results.

The aim of antibody reduction therapies in renal transplantation is to achieve a negative crossmatch by the time of transplantation, although in practice current therapies are often unable to reduce antibody titres sufficiently to achieve this goal. The HLA mini-columns were able to reduce microbead MFI values by around 90% on average, this level of depletion should lead to a significant reduction in RMF in the FCXM.

Figure 8.5 shows the FCXM data on the six cases highlighted earlier which were passed through the HLA-A2 mini-column. Pre-column, post-column, and peak elution fractions for each case were crossmatched against two separate lymphocyte preparations;

Cell 1 HLA type; HLA-A2; B35,55; C4,9; DR1,4; 52; DQ5

Cell 2 HLA type; HLA-A2,3; B8,62; C7,9; DR13,17; 52; DQ2,6

All six samples were positive against both cell types with RMF values for pre-column samples ranging from 2.3 to 21.3. Following mini-column depletion all samples fell below our RMF cut-off of 2.3 and in the clinical setting would have been called crossmatch negative.

The RMF values observed differ between the two donor cells used for FCXM, with values considerably higher for cell line 1. The increased sensitivity of cell type 1 is presumably due to the HLA type of the cell as cell 1 is homozygous for HLA-A2, whereas cell 2 was typed as HLA-A2,3.

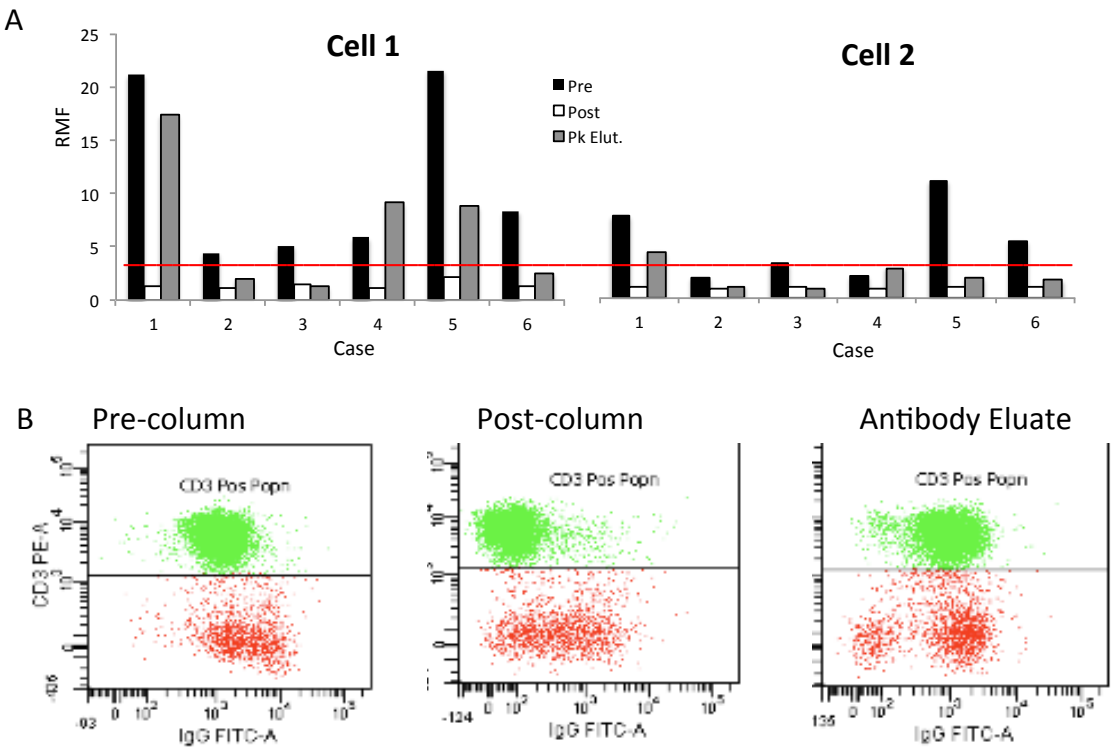


Figure 8.5: **Flow cytometry Crossmatching (FCXM) of samples treated with HLA columns, and reactivity and eluted antibodies.** A) Relative median fluorescence (RMF) from pre-column, post-column, and peak elution fractions for HLA-A2 column cases 1-6. Positive cut-off RMF of 2.3 is indicated by the red bar. B) Flow cytometry dot plots showing the reduction in cell binding post-column and the restoration of anti-donor reactivity in the antibody elution fraction. The FCXM technique in outlined in greater detail in section 2.6.4.

The same donor cells were tested against the same panel of sample by CDC analysis. Of the six cases, cases 1 and 5, the two highest positive flow cytometry crossmatch samples, were the only samples to test positive on the pre-column sample.

In both cases the CDCXM result on the post-column sample was negative, with CDCXM positivity restored when testing the peak column elution sample (Figure 8.6).

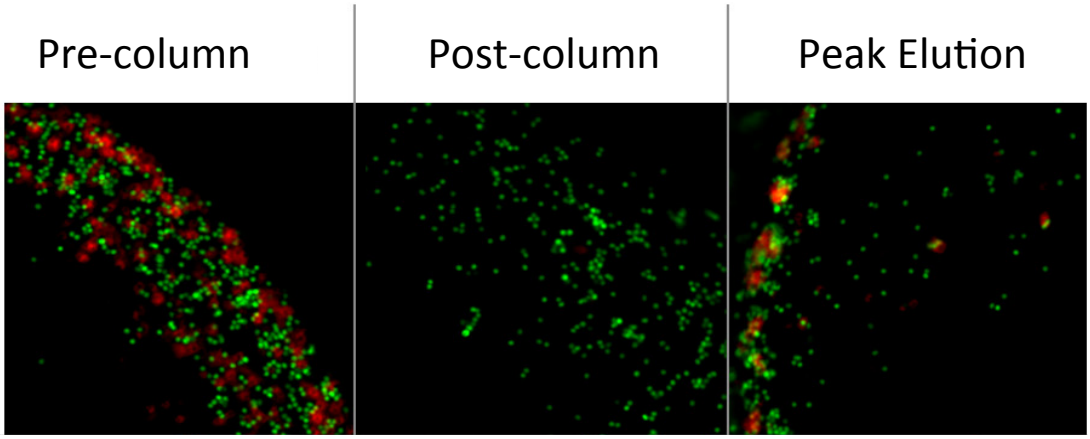


Figure 8.6: CDCXM data showing effect of column depletion from Case 5. Positive crossmatch with pre-HLA-A2 column treated sera, rendered negative by column absorption. CDC reactivity is then restored in the eluate. Red stained cells denote cell lysis and CDC positivity

The crossmatch data clearly shows the potential of the HLA mini-column to reduce HLA-specific antibody to levels sufficiently low as to change the pre-transplant crossmatch from positive to negative. The following section shows experimental data from a clinical scale HLA antibody removal column.

8.3.6 Clinical Scale HLA-Column Validation

Based upon earlier column capacity analysis using the 1ml mini-columns we estimated that the potential binding capacity of a 50ml clinical scale column containing approximately 50mg of sHLA would be in the region of 12.5 to 15mg of HLA specific

antibody. This calculation is derived from a column capacity in the region of 250μg/ml as shown in figure 8.2.

Preparations of W6/32 and the HLA-DR specific monoclonal antibody L243 were standardised to 110mg/litre and 120mg/l respectively. Two hundred and fifty millilitres of W6/32 preparation was passed through the clinical scale HLA-A2 column at a flow rate of 50ml/min and eluted in 1 litre 100mM glycine pH11.0. Similarly, 250ml of L243 (120mg/l) was passed through the clinical scale HLA-DR11 column at 50ml/min then eluted in 100mM glycine pH11.0. Column capacity was calculated by measuring the total protein content of the eluate from each column run (figure 8.7).

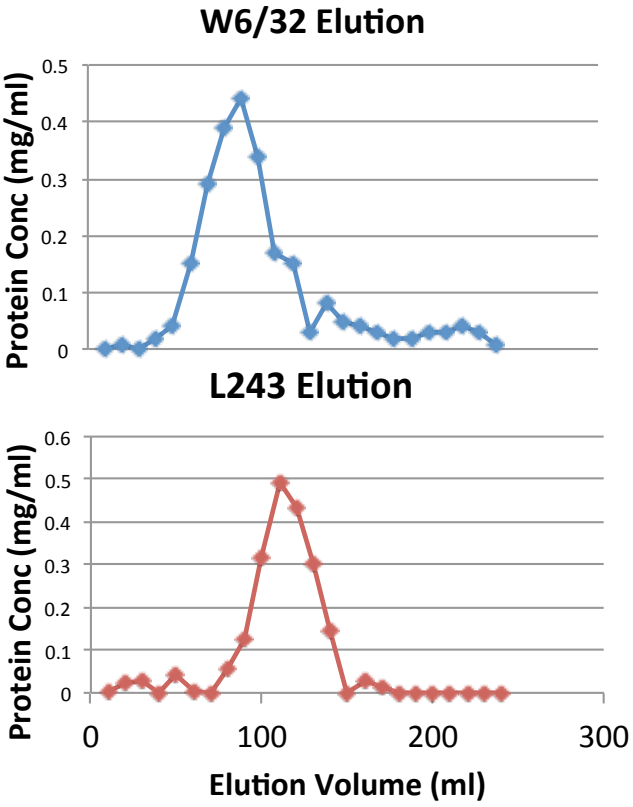


Figure 8.7: Estimated column capacity using saturating concentrations of HLA-specific monoclonal antibodies. HLA-A2 column estimated capacity 24.1mg, HLA-DR11 column estimated capacity 20.7mg

Calculation of the area under the curve provides an estimated column capacity for the HLA-A2 column of 24.1mg, and for HLA-DR11 of 20.7mg. These values are above the projected column capacities based upon 1ml column capacity.

8.3.7 Clinical Scale HLA Column – Patient Samples.

Three patients were chosen with HLA-A2 specific reactivity and three were chosen with HLA-DR11 specific antibody as confirmed by luminex single antigen bead analysis. The plasma effluent samples were centrifuged at 4000g for 30 minutes to pellet any preformed cryogel and other cellular debris then diluted to a total volume of between 2-2.5 litres (approximately the plasma volume equivalent to a 50-60kg patient) using fresh frozen plasma tested negative for HLA-specific antibody (data not shown). Starting volumes and HLA-A2/DR11 specific MFI values are shown in Table 8.1.

Table 8.1: Starting MFI levels for samples prepared for clinical scale column depletion. Samples were diluted using fresh frozen donor plasma previously tested to confirm negativity for HLA-specific antibodies.

Sample	Volume (ml)	A2 MFI
LT66	2000	5899
LT68	2000	6904
LT79	2000	18151
		DR11 MFI
LT56	2200	4798
LT65	2000	2742
LT45	2000	6580

8.3.8 Clinical Scale HLA Column-Circuit Design

The clinical scale column circuit was set up as outlined in Figure 2.16. The circuit incorporated sampling points prior to treated sample collection so that column performance could be monitored at regular intervals throughout the treatment run.

Prior to the first patient sample run a blank column containing 50ml of unbound 'naked' sepharose was set up and 2 litres of HLA antibody negative diluted FFP was run through the column on a continuous loop for three hours to monitor the pressure at each of the three pressure monitoring points built into the circuit. Figure 8.8 shows the pressure monitoring data over three hours.

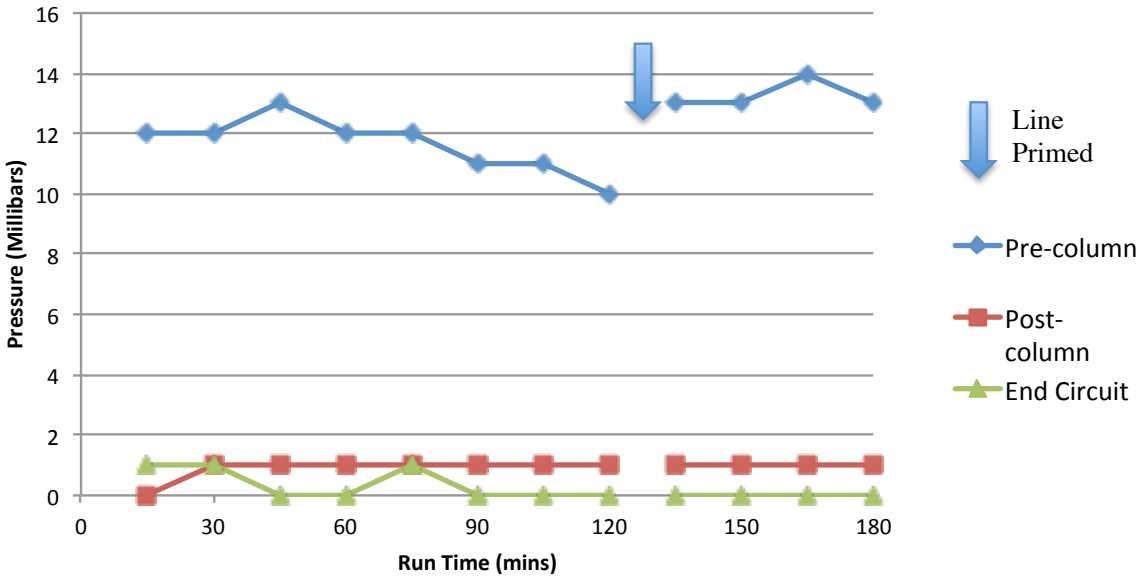


Figure 8.8: Clinical scale column circuit testing. Plasma was run continuously through the blank column for three hours. Pressure readings remained constant throughout the procedure.

Over the three hour period of pressure monitoring the pressure remained constant at each sampling point of the circuit. It was necessary to 'prime' the line to remove air that had built up in the system after around two hours of running time. The pressure readings were well below the estimated crush pressure of the sepharose, which is thought to be in the region of 100 millibars. This data enabled us to proceed to patient samples with confidence that the circuit would be robust enough.

8.3.9 HLA-A2 Clinical Scale Column Depletion

Three patients were run through the HLA-A*02:01 column. The MFI values were measured pre-treatment, post-treatment, and at various intervals during the course of treatment. Eluted antibody was also purified, concentrated and assessed for correct specificity, soluble inhibition experiments using panels of purified soluble HLA proteins were used to determine epitope specific reactivity of column eluted IgG. Results for each of the three patients will be presented individually.

Patient 1 LT66 : Column Performance

Plasma effluent from LT66 was diluted to 2 litres in HLA antibody negative fresh frozen plasma (FFP) and A2 reactive MFI of 5899 was measured by single antigen bead assay (SABA). The patient sample was connected to the column circuit as outlined in Figure 2.6. Following a single pass through the clinical scale column A2 reactive MFI had fallen to 1167, a reduction of 80.22%. Figure 8.9 shows the reactive MFI values measured at defined timepoints throughout the depletion cycle, samples were

taken every 100ml of patient plasma and selected samples were tested by microbead analysis. Starting values are shown at timepoint 0.

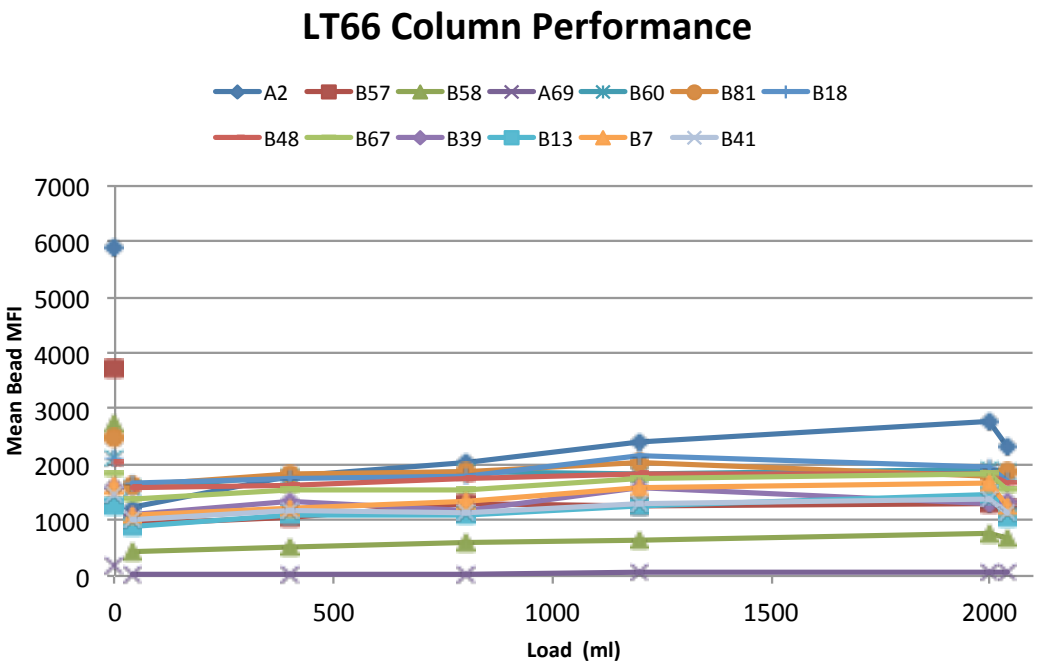


Figure 8.9: Depletion of HLA-A2 specific reactivity in patient LT66. Direct measurements of MFI reactivity are taken at regular timepoints throughout the depletion cycle.

HLA-A2 reactive MFI values are shown to gradually increase as the cycle progresses, presumably as the column increases in saturation and specific antibody binding reduces in efficiency.

Following column depletion captured antibody was removed via alkaline elution using 100mM glycine pH11.0. Figure 8.10 shows the eluted MFI values at selected time-points in the elution cycle.

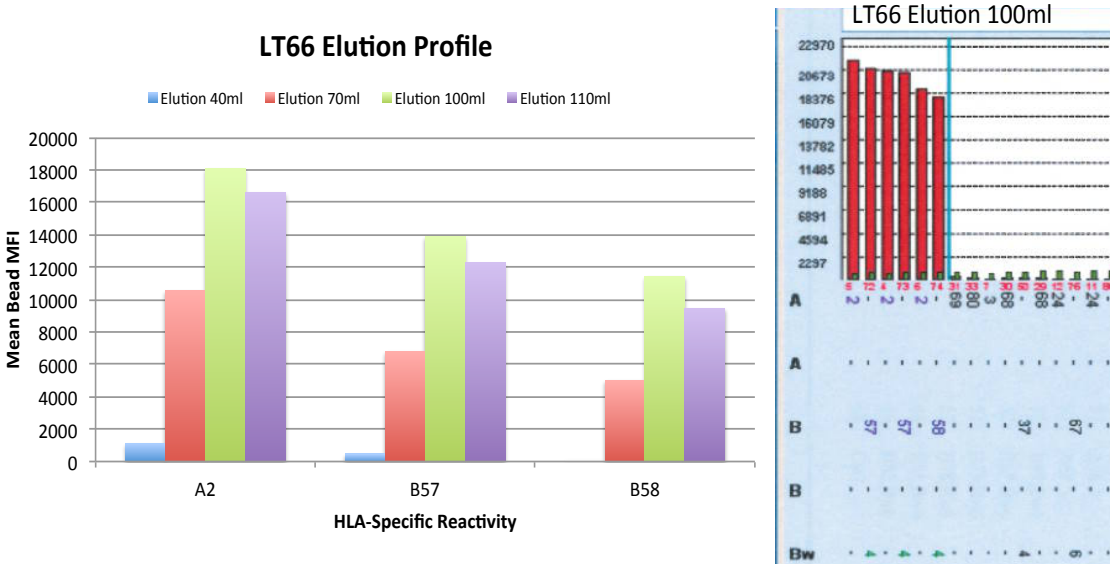


Figure 8.10: Analysis of antibody eluted from the column following treatment of patient LT66. Bead reactivity of purified HLA-specific antibody at different timepoints in the elution cycle (left panel). Single antigen bead analysis of 100ml timepoint of elution cycle (right panel).

Elution fractions of 10ml each were collected and protein content was measured using OD280 readings. A total of 14.4mg of HLA specific antibody was isolated from the clinical scale column.

Patient 2 LT68: Column Performance

LT68 plasma effluent was diluted to 2 litres in FFP to give anti-HLA-A2 reactivity of 6904 (table 8.1). Patient sample was then passed through the clinical scale column at 40-50ml/min. Reductions in specific MFIs are shown in figure 8.11. Following column treatment the A2 reactive MFI reduced from 6904 to 840. In addition

anti-B57 and anti-B58 reactivity reduced from 7805 to 1295, and 4793 to 539 respectively. Each of these represents a percentage reduction in MFI in excess of 80% in a single column treatment (figure 8.11 inset).

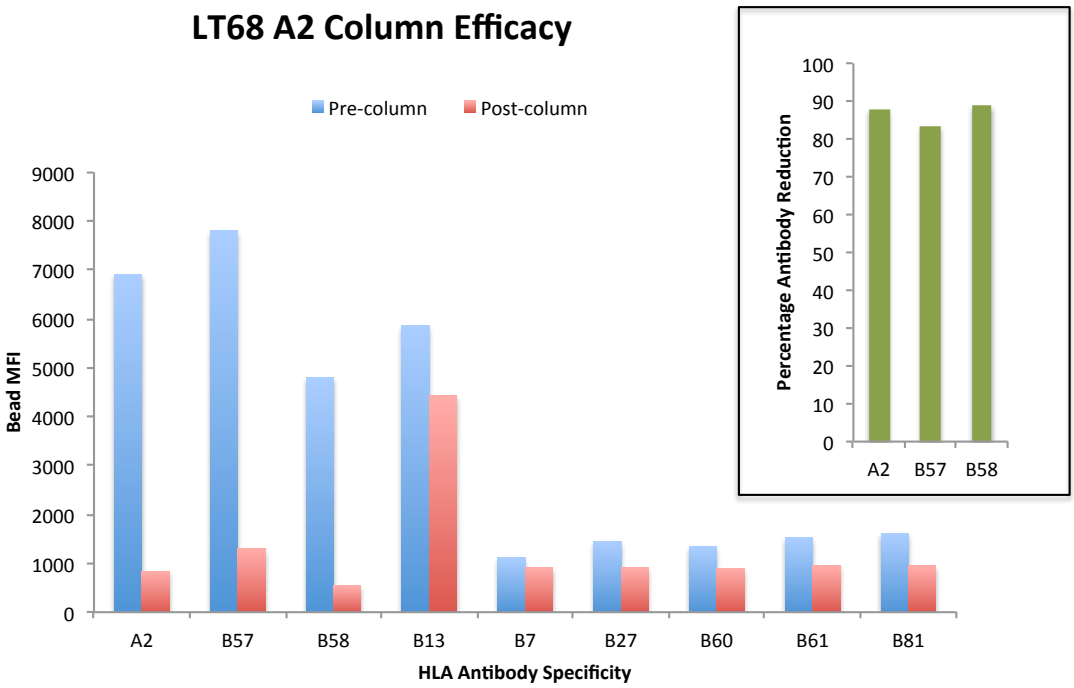


Figure 8.11: LT68 column depletion effectiveness. Post-column MFI values show effective reduction from pre-column levels, equating to an overall reduction in excess of 80% (inset).

The effectiveness of the column removal was monitored by measuring samples at specific points during the removal cycle. The levels of the relevant antibodies throughout the cycle are shown in figure 8.12. The clinical scale column is extremely efficient at removing antibody specifically with selected third party specificities (HLA-B7,13,27,60,61,81) being left with reactivity left mainly intact.

Figure 8.12 shows column removal for HLA-A2 shared epitope specific antibodies are depleted rapidly as the depletion cycle begins, and remain low throughout

the entire depletion cycle. This suggests that although the column removes over 80% of HLA specific reactivity in a single pass the column does not appear to have reached saturation point.

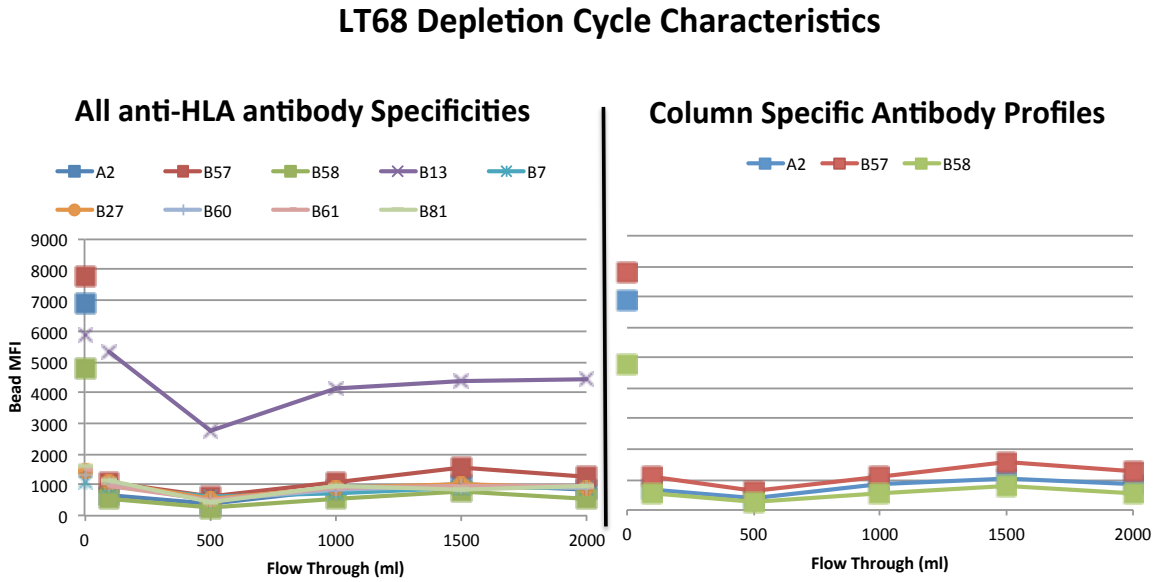


Figure 8.12: MFI values recorded during the antibody depletion cycle. Column specific and third party specificities (left panel); Column specific antibodies only (right panel)

Antibody was eluted using 1 litre of 100mM glycine pH11.0 and elution profiling was performed by measuring the OD280 values of protein absorbance at 20ml intervals. Peak elution was observed at 100ml elution buffer, with the MFI values for each eluted antibody specificity shown in figure 8.13.

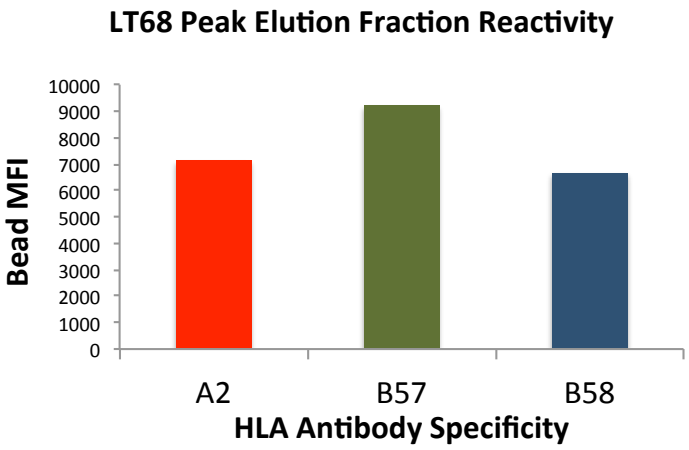


Figure 8.13: MFI values for eluted antibodies. Peak elution fraction MFI values specific for HLA-A2, B57, B58 representing the 62GE epitope.

Patient 3 LT79: Column Performance

LT79 plasma effluent was diluted to 2 litres in FFP to give anti-HLA-A2 reactivity of 18151 MFI (table 8.1), therefore this patient represented a much higher MFI than the previous two cases. MFI values such as this are seen frequently in our sensitised patient cohort but are of sufficiently high level to potentially prevent the entrance into our HLA antibody incompatible (HLAi) transplant programme.

Once again the sample was passed through the clinical scale column at a rate of 40-50ml/minute. Column specific antibodies; anti-A2, A69, B57, and B58 (detailed epitope definition for this patient was described in section 4.3.2) showed reduction after one cycle of depletion as follows: anti-A2 18151>8547, anti-A69 10020>2765, anti-B57 12201>2091, anti-B58 9940>786 MFI. Although each of these reductions were substantial (>50% for A2, >75% for A69, B57 and B58), there remained considerable reactivity in the post-column sample. Indeed, the MFI values observed following the

first depletion cycle were then similar to the MFI values at starting point for the previous two patient samples tested. Therefore, following elution of HLA specific antibody from the clinical scale column (details to follow) the post-column fraction was passed through the column for a second depletion cycle with flow rates mirroring those utilised for the first passage. Following the second round of depletion the specific reactivities were as follows: anti-A2 5141, anti-A69 1254, anti-B57 922, anti-B58 329 MFI. Figure 8.14 details the reduction in MFI of each of these antibody specificities for each depletion cycle along with the calculated percentage reduction in MFI.

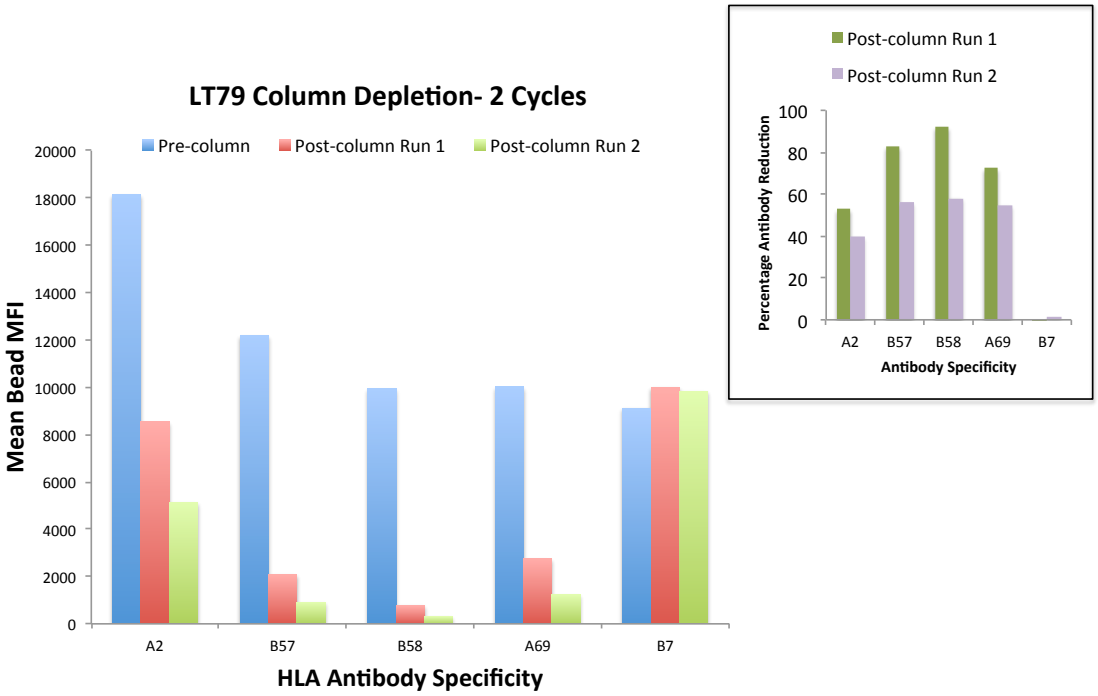


Figure 8.14: LT79 antibody depletion. Column specific antibody (A2,A69,B57,B58) depleted with 2 depletion cycles. Non-column specific HLA-B7 antibody was not reduced. Inset: Percentage depletion of antibody reactivity

The statistics suggest that column depletion has been inferior to that observed with the previous two cases, where A2 depletion was observed at >80% on the first pass

compared with just over 50% in this instance. However, it must be considered that the antibody levels are much higher in this patient, consequently it is a very clear possibility that the microbead assay had exceeded the saturation point for our column specific antibodies, in particular antibody against HLA-A2 (see chapter 3 for more detailed explanation). Therefore the microbead analysis for this patient was repeated at a dilution factor of 1 in 10 for pre-column, post depletion cycle 1, and post-depletion cycle 2 samples. The resulting revised figures for column depletion are shown in figure 8.15.

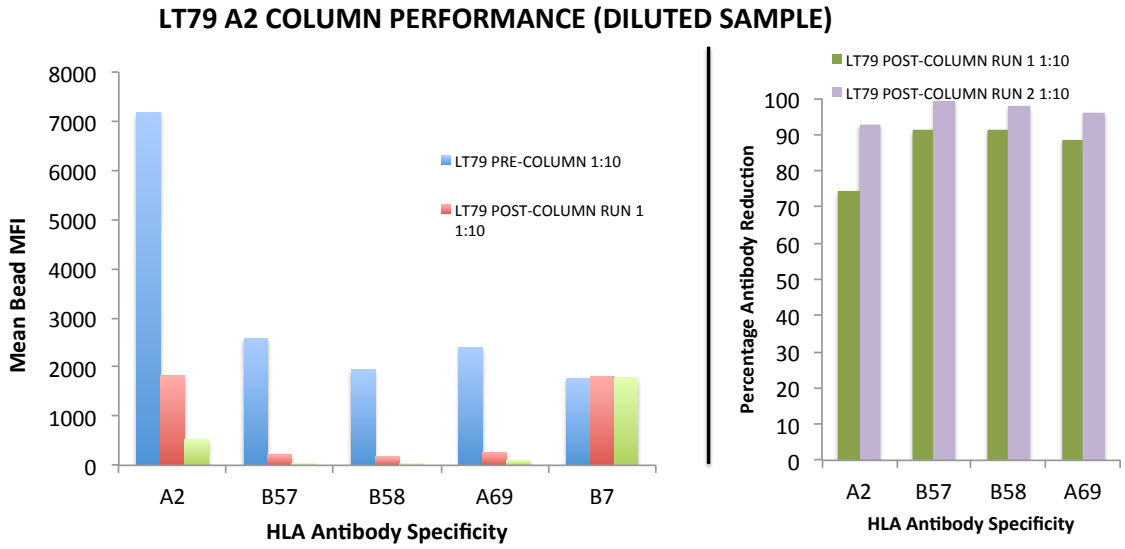


Figure 8.15: Dilution analysis of patient LT79 saw greatly enhanced column efficiency when sample tested at 1in10 dilution. Left panel: reduction in raw MFI values. Right panel: percentage reduction in reactivity after each depletion cycle

These data clearly show that on the initial microbead analysis the assay was indeed saturated and that the true scale of the depletion was greater than initially calculated with a reduction in anti-A2 reactivity of 75% after one pass rising to >90%

following a second round of depletion. After two depletion cycles almost all of the anti-A69, B57 and B58 reactivity has been removed.

The analysis of column depletion through the course of the depletion cycles shows the high titre of the antibody leading to a plateau of antibody depletion at around 1 litre of plasma volume passed through the column (Figure 8.16). HLA-A2 microbead reactivity is immediately reduced from its starting value of over 18,000MFI to around the 5,000 MFI level as plasma begins to flow distal to the column. MFI values then increase up to around 9500 whereafter the antibody level remains constant.

The specificity of the column is again highlighted by considering the MFI values of the HLA-B7 microbead which remains constant, in the region of 9000-10,000MFI during the entire duration of the two antibody depletion cycles.

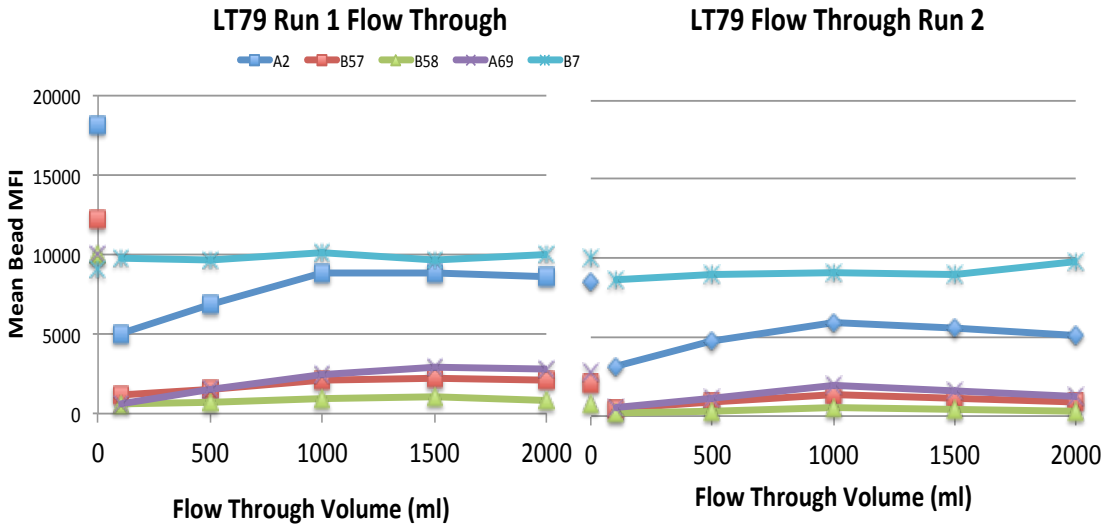


Figure 8.16: Microbead analysis of HLA specific antibody through two depletion cycles. High titre HLA-A2 specific antibody appears to saturate the column at around 1 litre of plasma treated.

Figure 8.16 also shows the effectiveness at removing the antibody reactive to epitopes shared by HLA-A69, B57, and B58. These specificities are drastically reduced by the first column depletion. Reactivity for these specificities is initially in the negative regions then begins to rise gradually through the course of the first depletion cycle. Although HLA-A69, B57, and B58 MFI values are low they are still competing for binding sites on the A2 column in very close proximity to other A2 epitope specific antibodies present in much higher titre. The data also suggests that a further cycle of depletion would be required to fully deplete HLA-A2 specific reactivity.

As before bound antibody was removed from the column following each depletion cycle using alkaline elution. Twenty millilitre fractions were taken through the elution cycle and each fraction was assessed for protein content using the nanodrop OD280 method. For each depletion cycle the elution fraction from 80-100ml contained the highest protein concentration. This fraction was then chosen for single antigen microbead analysis. The MFI values for each of the column specific eluted antibodies are shown in figure 8.17.

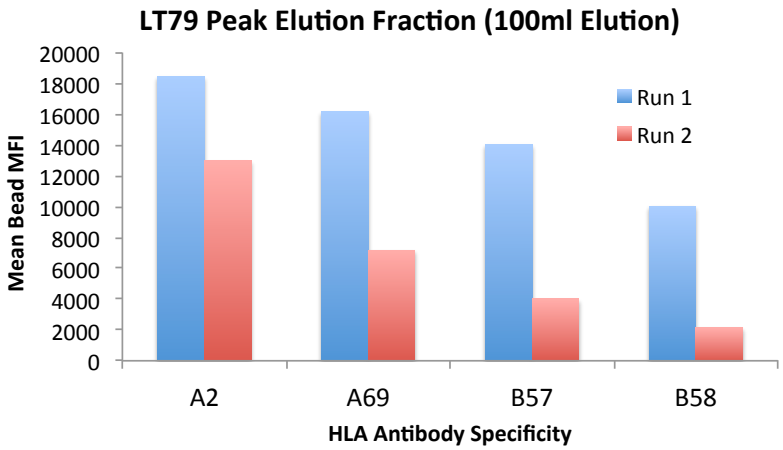


Figure 8.17: MFI values for eluted antibodies for patient LT79. Peak elution fraction (100ml elution fraction) tested for each depletion cycle.

High values were recorded for all specificities of antibody eluted after depletion cycle 1. HLA-A2 specific reactivity measured in excess of 18,000 MFI. Similarly HLA-A69, B57, and B58 reactivities measured 16,000, 14,000, and 10,000 respectively. Following a second depletion cycle once again there were significant concentrations of HLA specific antibody eluted from the column, in particular anti-A2 detected at >12,000 MFI, suggesting that once again the column had reached saturating levels. This is supported by the fact that an HLA-A2 MFI of 5141 was still detectable in the patient sample even following two rounds of column depletion. In contrast the MFI values of the other column specific eluted antibodies, HLA-A69, B57, and B58 were eluted in much lower concentration after the second depletion. This suggests that only residual low level reactivity remained in the patient sample, a fact borne out by the corresponding MFI values in the post-column patient sample (HLA-A69: 1254, B57: 922, B58: 329MFI).

8.3.10 HLA-DR11 Clinical Scale Column Depletion

As was performed for the HLA-A*02:01 column, three patients were run through the HLA-DRB1*11:01 column. The circuit design, flow rates, elution conditions and regeneration protocol were all performed identically to the HLA-A*02:01 column depletion protocol. The MFI values were measured pre-treatment, post-treatment, and at various intervals during the course of treatment. Eluted antibody was also purified. As for HLA class I column depletion results for each of the three patients will be presented individually.

Patient 1 LT56: Column Performance

Plasma effluent taken from the first round of double filtration plasmapheresis prior to transplant for patient LT56 was diluted to a final volume of 2.2 litres using fresh frozen plasma. Microbead analysis of the diluted sample gave HLA-DR11 reactive raw MFI of 4798 (Table 8.1). The entire sample was then passed through the HLA-DR11 column at a steady flow rate of 40-50ml/min, with samples taken throughout the cycle to assess column performance. Figure 8.18 shows the depletion of HLA-DR11 reactivity and also the marked depletion of reactivity for a range of other HLA-DR specific beads.

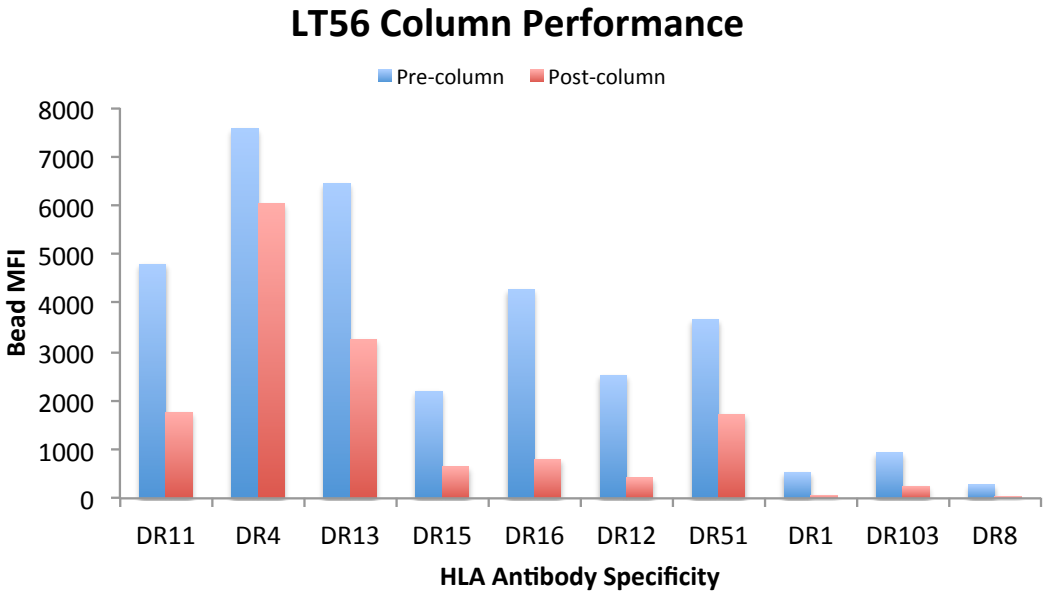


Figure 8.18: HLA-DR11 column depletion for patient LT56. Pre and post-column microbead MFI values for a range of HLA-DR specificities.

Raw MFI for HLA-DR11 was reduced from 4798 to 1762 a percentage fall of 63%. However as figure 8.18 demonstrates, the HLA-DR11 column was able to very

effectively deplete a range of HLA-DR bead reactivities. Raw MFI values for HLA-DR12, DR15, DR16 and DR51 were reduced by 83%, 73%, and 81% respectively. Specificities with markedly lower starting levels: HLA-DR1,103 and 8 were reduced by even larger percentages, 89%, 79%, and 94% respectively. Indeed, of the ten bead specificities listed in figure 8.18 only HLA-DR4, DR13, and DR51 raw MFI values were less effectively depleted than the HLA-DR11 MFI.

The analysis of the column removal measured during the depletion cycle shows HLA-DR11 reactivity is reduced drastically as the first few millilitres flow through the column (figure 8.19). As the depletion cycle continues the DR11 bead MFI rises steadily as the column presumably becomes more and more saturated.

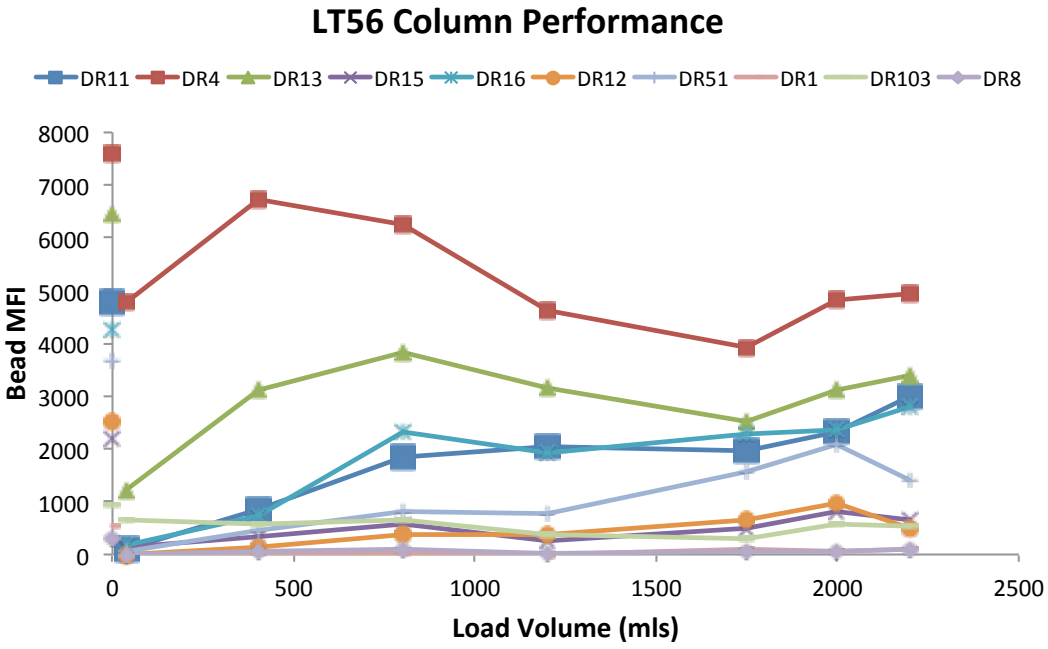


Figure 8.19: Antibody levels monitored through the depletion cycle. HLA-DR specific reactivity falls for a number of specificities presumably due to extensive epitope sharing.

Figure 8.19 also shows very similar removal dynamics for a range of other HLA-DR specificities. MFI values for the specificities listed in figure 8.19 all drop significantly in the early portion of the depletion cycle and follow the trend described for HLA-DR11 typically leading to MFI values around 50% of starting MFI when the final sample point at the end of the cycle is tested. The two notable exceptions to the trend are the MFI values corresponding to HLA-DR4 and HLA-DR13. These specificities appear to reduce initially then pass through in greater concentration for around the first 400ml, then MFI values fall again presumably as antibody is absorbed by the column. This continues until around 1750ml has passed through before a second rise in MFI to complete the depletion cycle. Possible explanations for these observations will be given later.

The column bound antibody was then eluted in the same way as before, by flow through of 1 litre 100mM glycine pH11 with 10ml fractions being taken throughout the elution cycle. Figure 8.20 shows the single antigen microbead data for all of the antibody specificities eluted from the column. In addition to the peak elution fraction (110ml as determined by OD280 protein quantification), both 40ml and 70ml elution fractions were also analysed by microbead testing.

High levels of HLA-specific antibody were eluted from the HLA-DR11 column. At peak elution HLA-DR11 bead reactivity reached an MFI of 13459, which was surpassed by a DR13 peak reactivity of 16165. Interestingly, despite only a modest post-column reduction in MFI for HLA-DR4 reactivity (7581 down to 6049) large amounts of DR4 specific antibody was eluted (peak MFI: 12280). This suggests the presence of multiple antibodies present in the original sample, which would appear to be specific for a number of epitopes on the DR4 molecule. Some of these presumably share epitopes with the DR11 molecule but others do not- as suggested by a post-column DR4 specific MFI of 6049 and a percentage reduction of just over 20%.

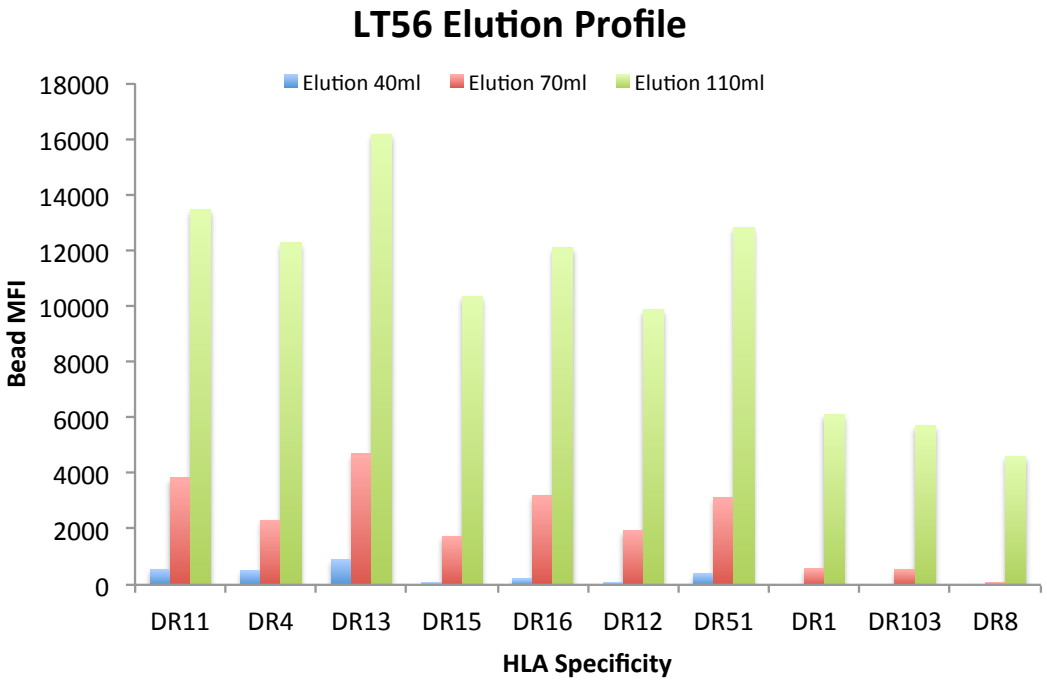


Figure 8.20: LT56 column specific antibody elution. A range of antibody specificities were eluted from the DR11 column.

Patient 2 LT65: Column Performance

Plasma effluent from patient LT65 was diluted to 2 litres using fresh frozen plasma to give a HLA-DR11 reactive MFI of 2742. The sample was then passed through the 50mg HLA-DR11 column as before. Figure 8.21 shows the fall in DR11 MFI post-column from 2742 down to 703 a reduction of 74.4%. A number of other specificities were also reduced by a similar magnitude; DR8 down by 73.8%, DR12 down 53%, DR13 down by 65.8%, DR14 down by 67%, DR15 and 16 down by 77.9 and 75.1% respectively. Other specificities such as DR4, DR17, and DR18 were also reduced, but by much lower magnitudes (18.7, 36.3, and 32.7% respectively). Pre-

column MFI levels for HLA-DQ7,8, and 9 were completely unaffected following column depletion.

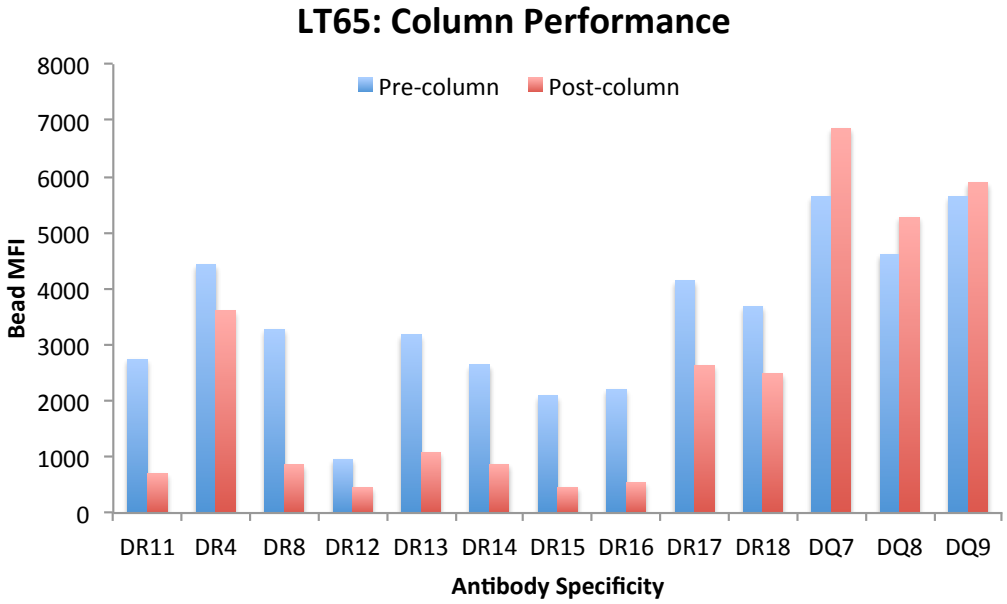


Figure 8.21: Reduction of HLA reactivity in patient LT65. Pre and post-column microbead MFI values for a range of HLA-DR are depleted. HLA-DQ specificities remain unchanged

The time-course analysis of bead reactivity during the depletion cycle is shown in figure 8.22. A number of specificities show a significant early reduction in MFI from starting level (Figure 8.22A) which then reach a plateau following around 500ml of plasma treated. It should be noted that the very early reduction in MFI is due mainly to the dilution effect of switching from PBS running through the column to plasma as indicated clearly by the initial reduction in HLA-DQ7,8,9 reactivity which rapidly stabilises at pre-column levels after around 200ml of treated plasma.

It can be seen in figure 8.21 that some specificities, for example DR11, 8, 13,14,15,16 are reduced by much greater magnitudes than others (DR4,17,18). As

mentioned earlier this is presumably due to the presence of multiple epitope specific antibodies, some of which are present on the DR11 protein. This appears to be a particularly common occurrence of antibodies directed against HLA-DR4 as this was also observed in patient LT56.

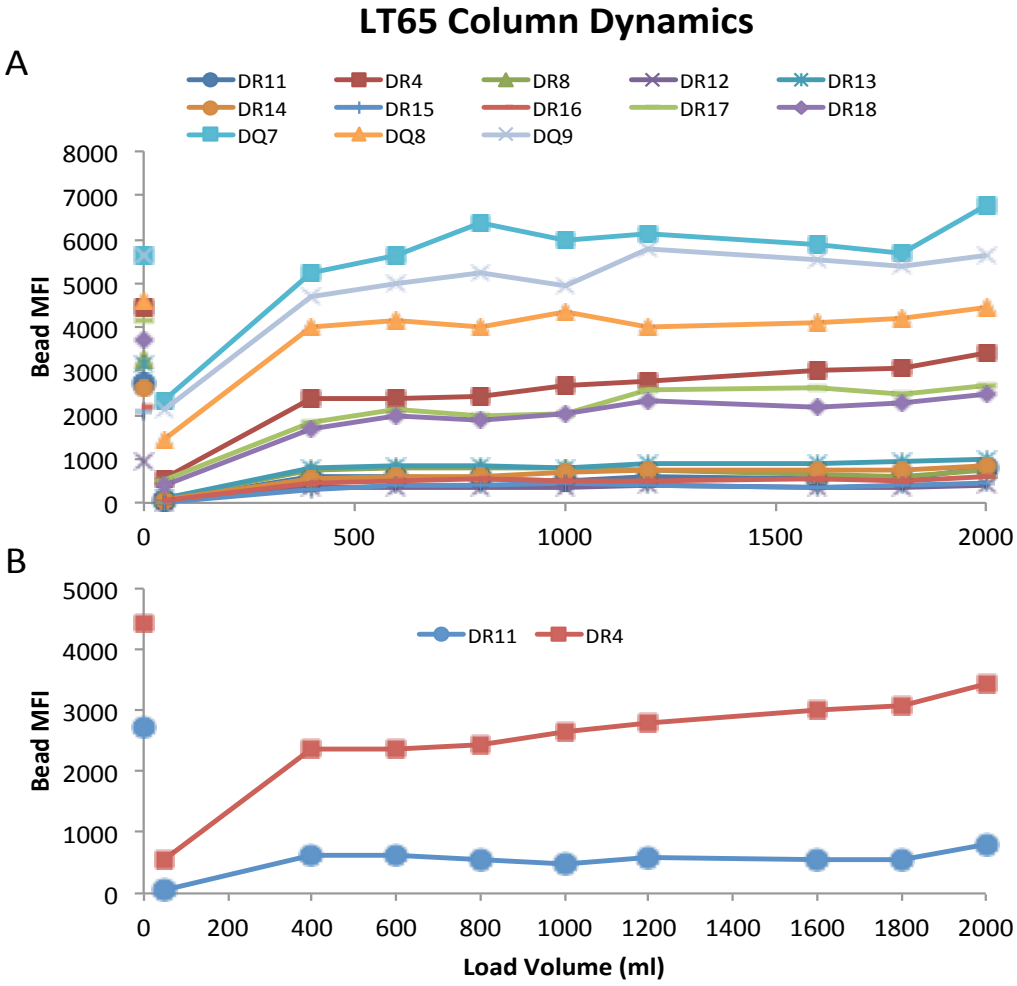


Figure 8.22: HLA-DR11 column dynamics for depletion of patient LT65. A) MFI values of antibody specificities through the depletion cycle. B) HLA-DR11 and DR4 bead specific MFI values

Figure 8.22B highlights the difference in MFI reduction between these two groups of antibodies by directly comparing the profiles of both HLA-DR11 and DR4

reactivity. HLA-DR11 antibody was measured at a relatively low level of <3000 MFI prior to treatment which is then reduced to well below 1000MFI upon commencing the depletion cycle. Reactivity then remains at low levels throughout the entire treatment, remaining below 1000MFI throughout. Conversely, HLA-DR4 reactivity is initially reduced by around 50% following the first 400ml of plasma treatment as the HLA-DR4 reactive component which share one or more epitopes with DR11 are absorbed by the column. Reactivity then increases steadily through the duration of the cycle as the column becomes increasingly saturated with DR11 epitope specific antibody. As before the bound antibody was then eluted from the column using glycine pH11.0 collected in 10ml fractions and assessed for protein content using OD280 readout. Figure 8.23 shows the eluted antibody profile at selected fractions using single antigen microbead analysis.

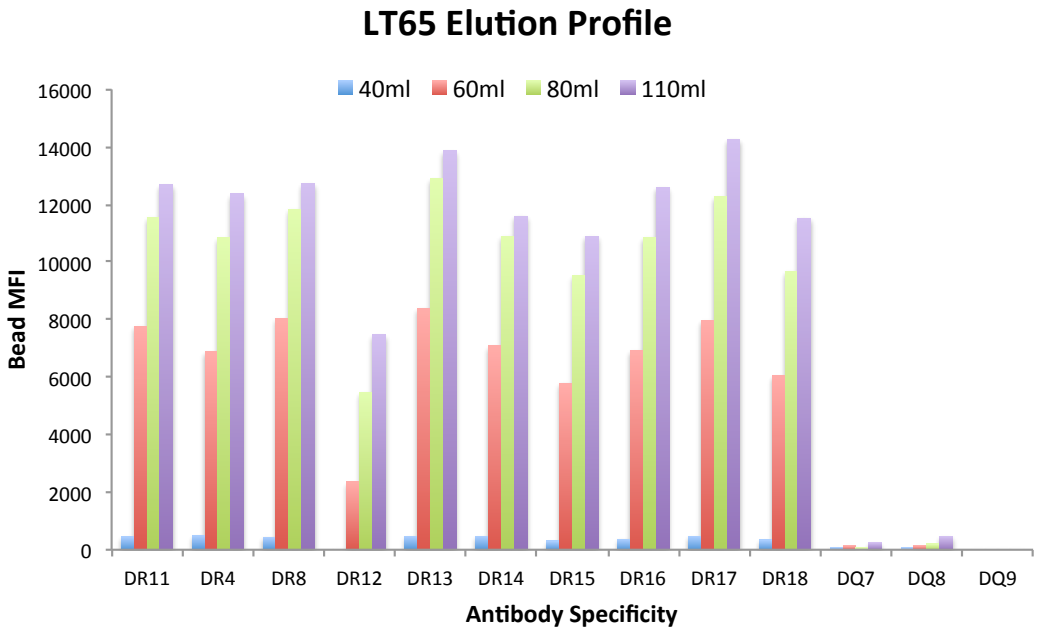


Figure 8.23: HLA-specific antibodies eluted from HLA-DR11 column for patient LT65. Samples tested by microbead analysis at key points in the elution cycle.

It can be seen from figure 8.23 that large amounts of all specificities that had previously shown MFI reduction could be eluted from the DR11 column. With the slight exception of DR12 the MFI values across the whole range of HLA specificities detected are extremely consistent, taking the 110ml elution fraction as an example and omitting DR12, the lowest detected MFI is 10889MFI for DR12 rising to 14226 for DR17. This observation suggests that the epitope composition of the antibodies reacting with these beads is probably identical and that the differences in MFI observed can be attributed to the variation in antigen density on each of the beads.

It is also of interest to note that DR17 gives the highest MFI reactivity upon elution as this specificity shows only a modest pre- to post-column reduction (4138>2635MFI).

Patient 3 LT45: Column Performance

As with the other patient samples plasma effluent from patient LT45 was diluted to a final volume of 2 litres using HLA antibody negative fresh frozen plasma (FFP) to give HLA-DR11 reactive MFI of 6580. Sample was run through the clinical scale column as before and the degree of HLA-specific antibody removal is summarised in figure 8.24. HLA-DR11 reactive MFI was reduced by 62%, MFI falling from 6580 to 2524. A range of other specificities was also reduced by between 60 and 80% suggesting the presence of a common reactive epitope, which will be analysed in more detail later.

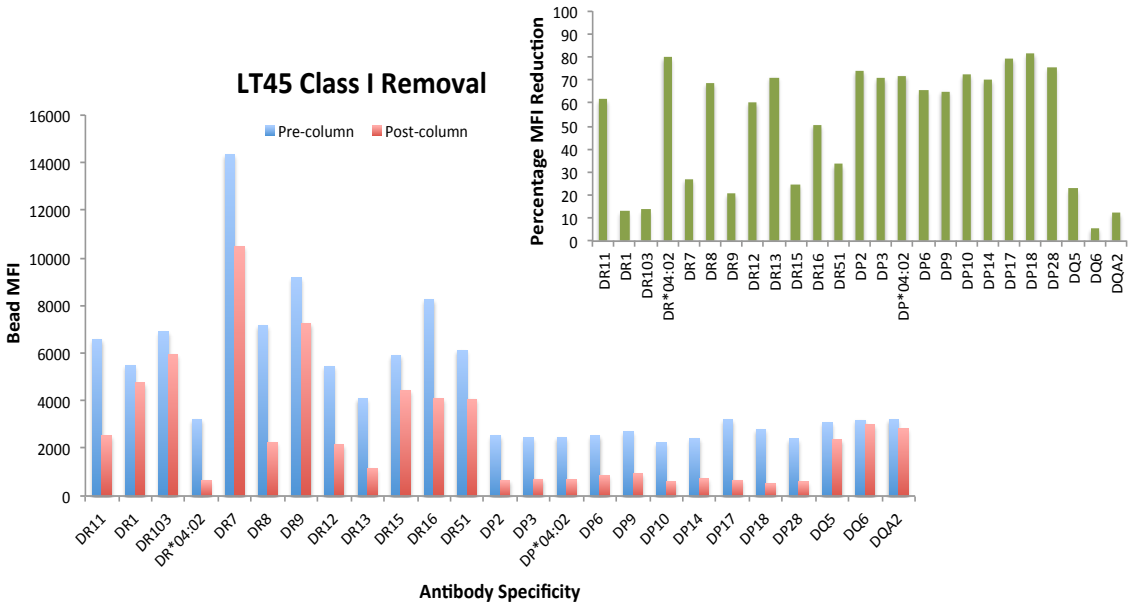


Figure 8.24: Patient LT45 antibody removal. HLA-DR11 reactive MFI reduced by 62%, a range of HLA-DP specificities reduced by between 65 and 82%

As observed with earlier examples antibodies specific for the HLA-DQ locus were unaffected by column treatment. In this instance antibodies were detected against HLA-DQ5 and DQ6 and also the DQ alpha chain (figure 8.24).

Analysis of column reduction in real-time as the depletion cycle progressed showed that many of the specificities detected pre-column are depleted effectively only over the first 400ml of plasma flow (Figure 8.25 top panel). This data supports the finding in figure 8.24 which shows many of the specificities are only partially depleted, 10-30% only in many cases highlighting the fact that there are many epitope specific antibodies present that are not expressed on the DR11 protein. HLA-DR11 specific reactivity (figure 8.25 bottom panel) shows that the column was steadily saturated by DR11 epitope specific antibodies, with detectable anti-DR11 reactivity rising steadily through the course of the depletion cycle.

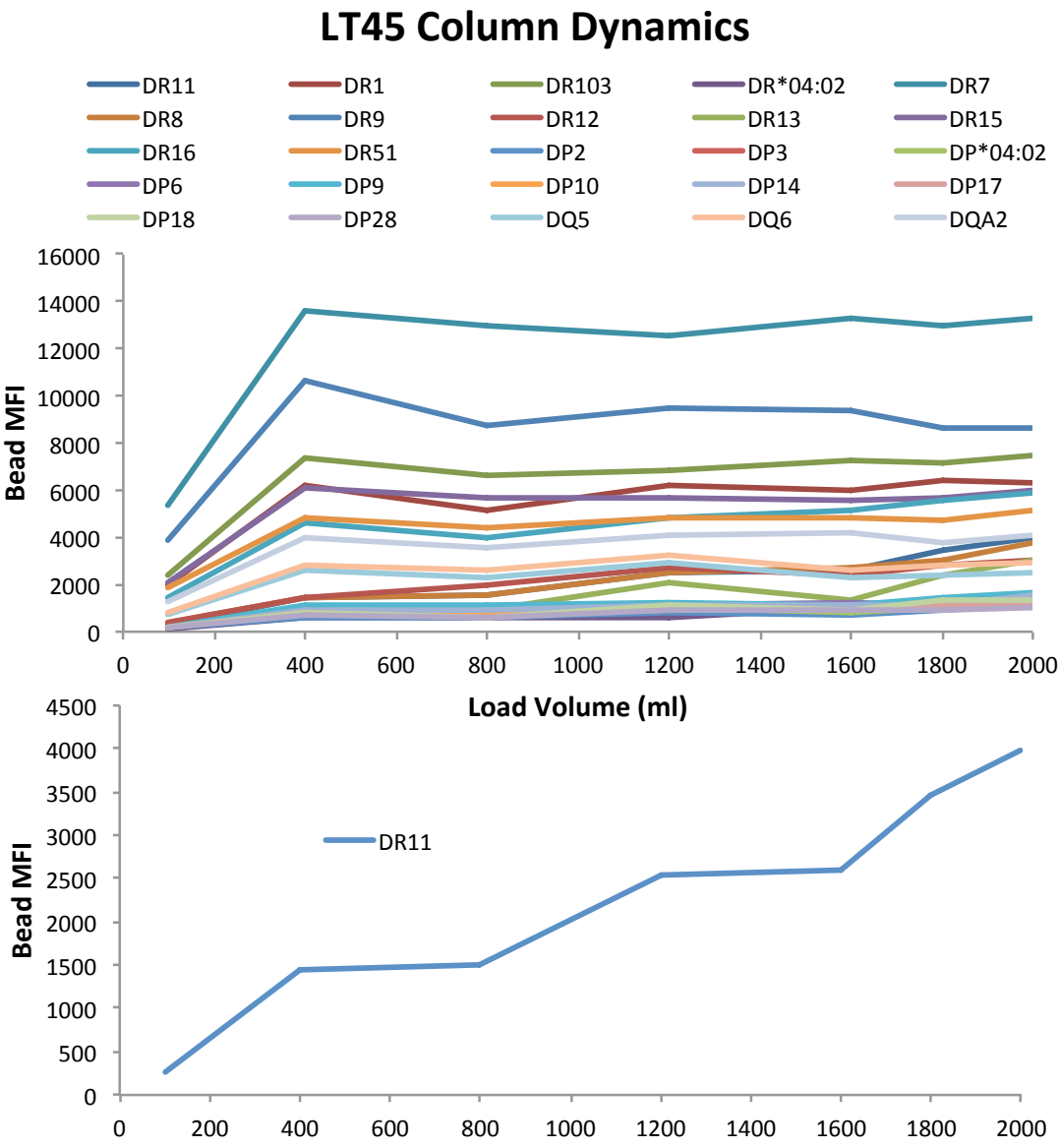


Figure 8.25: Antibody depletion through the column cycle for patient LT45. Many antibody specificities quickly reach saturation level (top panel). HLA-DR11 specific reactivity is reduced initially then increases steadily as binding capacity of the column is reduced.

Antibodies were then eluted from the column as before and specificity and titre was measured by microbead assay. Figure 8.26 shows the reactivities of all the positive beads in the microbead assay.

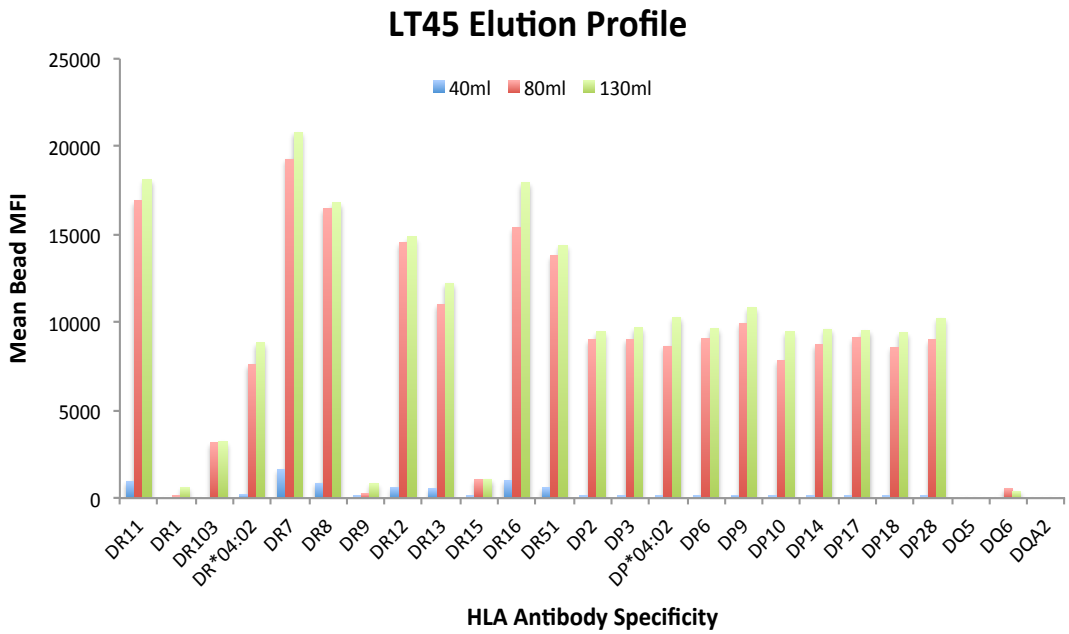


Figure 8.26: Specificities of LT45 patient antibodies eluted from clinical scale HLA-DR11 column. Samples were taken at key timepoints in the elution cycle and tested by microbead analysis.

It was immediately apparent that a range of antibody specificities had been eluted from the column indicating the presence of antibodies directed against numerous epitopes present on the DR11 protein. The effective elution of antibody directed against a range of HLA-DP locus proteins indicates the presence of antibody specific for the 70D/55D epitope. This common epitope is shared between DR11 and the HLA-DP specificities listed in figure 8.26. The amino acid positions of this shared epitope are shown in figure 8.27.

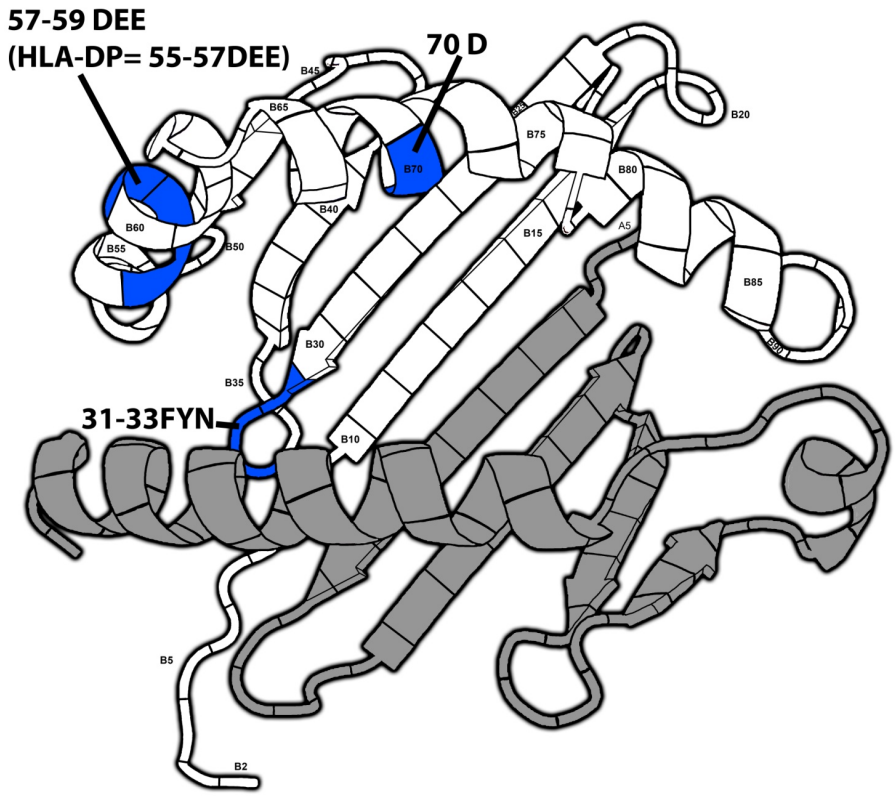


Figure 8.27: Ribbon diagram highlighting the position of the 70D / 57-59DEE epitope carried by HLA-DR11 and numerous HLA-DP specificities. Epitope positions on the beta chain of the class II HLA molecule are highlighted in blue.

8.4 DISCUSSION

Sepharose columns containing immobilized HLA protein provide a highly effective, selective, and specific method to absorb high titres of HLA-specific antibody from clinical scale preparation of human blood. HLA protein mini-columns and also clinical, large scale columns were highly effective at removing HLA specific antibody in an epitope specific manner. Highly specific antibody reduction, typically 50-80%, in a single absorption was routinely observed.

The use of HLA protein columns to specificially deplete HLA-specific antibodies may open up the possibility of transplantation to those patients with high titre broadly reacting antibodies. Patients who currently present with over 90% PRA are often unsuitable for HLA antibody incompatible transplantation. Indeed, a number of patients have failed to be accepted into our HLAi programme by virtue of excessive HLA-specific antibody titre.

The priniciple of these columns is identical to that of the Glycosorb columns used to selectively deplete ABO specific antibodies from patients prior to ABO-incompatible transplantation. As is the case with the columns used to remove ABO specific antibodies we aniticipate that specific HLA depletion columns will be much better tolerated by the patient and will not lead to a detrimental depletion of other serum proteins, such as clotting factors, and will also avoid issues involving hypotension observed with plasma exchange and DFPP[111]. In addition, the general maintenance of immune equilibrium so lead to a substantially reduced level of risk to the patient, particularly in the perioperative period.

The column approach compares very favourably with our experience of plasma exchange or double filtration plasmapheresis, both of which usually lead to overall antibody reduction of less than 50% with each round of treatment [91, 129, 130].

Antibody specificities that did not detect the epitope carried on the column protein were retrieved completely. Current protocols to reduce the levels of donor HLA-specific antibody prior to HLA incompatible transplantation have the major disadvantage of being non-specific, leading to a general comprising of overall humoral immunity. This study describes the use of soluble HLA proteins and their ability to inhibit the anti-HLA response in solid phase (mini-column and clinical scale column) format. The treatment of sera with HLA columns was highly effective in rendering highly positive donor/recipient crossmatches negative (figure 8.5). The fact that a strongly positive crossmatch was rendered negative following just a single round of HLA column depletion was highly encouraging as in the clinical settings it often requires mulitple round of DFPP to achieve a negative crossmatch, and in many cases we proceed to transplantation even in the presence of a reduced titre positive crossmatch [90].

Extensive testing has been carried out to demonstrate the structural integrity of the sHLA protein is maintained following multiple rounds of antibody depletion and column regeneration (figure 8.2) via the use of a monoclonal antibody directed against beta-2 microglobulin. This is important as the loss of protein from the column could be harmful to the patient and may have lead to increased levels of additional sensitisation.

This study has also highlighted the potential to reuse clinical scale HLA columns. We were able to effectively remove HLA-specific antibody from the column matrix leaving the HLA-protein intact. Any potential clinical use column would be expensive as a result of the high cost of GMP grade protein manufacture demanded by regulatory bodies so the option to reuse a particular column on the same patient following a regeneration protocol would provide enhanced cost-effectiveness.

The fact that patients are able react against a wide array of mismatch HLA epitopes does lead to difficulties when designing HLA protein columns. There are therefore two disctinct possiblities to be considered when attempting to design a colimn

for therapeutic use. In the study described here a single HLA specificity (HLA-A*02:01) was coupled to the column matrix and patients' were subsequently chosen on the basis of anti-A2 reactivity. In reality patients will have antibodies to many different and often multiple HLA specificities, therefore the decision must be made whether to produce a depletion column containing a panel of soluble HLA proteins, or to produce a 'bespoke' HLA column containing only the HLA specificities which correspond to the patients' HLA-specific antibody profile. In the case of a multiple antigen columbn careful consideration must be given to the composition of the HLA panel, with the aim being to optimise the number of proteins required to represent the widest array of potential epitopes. We have estimated that 30 separate class I antigens would be required to cover all of the currently identified epitopes. The obvious pitfall to this appraoch is that the individual proteins (and as a consequence epitopes) would be 'diluted' out as lower concentration of each specificity would need to be used. It may be prudent to deliberately omit certain epitopes which are only present on less common HLA antigens as these would be less likely to give rise to antibody formation.

The specific elution of HLA-directed antibodies from patient sera provides a valuable research tool. Many studies are ongoing to investigate the effects of HLA-specific antibody binding on various cell types linked to rejection of solid organ transplants. Recently, our group published data that indicated that the binding of HLA-specific antibody to cells of the human microvascular endothelium results in a significantly increased localised synthesis of complement component C4 [175]. This study, as with many other similar studies, used human serum containing donor cell HLA-specific antibody as the source of humoral stimulation. The common criticism of this approach is that it only *indirectly* suggests that HLA-specific antibody is the causative agent as there are a mulitude of other proteins that may be responsible for the effect observed. The use of purified HLA-specific antibody directed against donor HLA

will address this shortcoming and the fact that we are able to effectively quantify these antibodies can aid potential study design with the addition of antibody concentrations that reflect antibody concentration ranges seen *in vivo*.

Our group have published a series of studies describing the antibody responses involved in HLA incompatible transplantation. A key area of research remains to identify why certain individuals experience rejection episodes when faced with rising DSA levels and others do not. The ability to select and isolate these antibodies directly will provide us with a valuable tool with which to study various aspects of the humoral response. We will be able to determine the isotype of a particular antibody specificity, and also gain valuable insights into potential variation in antibody affinity and glycosylation.

This data provides encouraging evidence that a direct assay to identify anti-idiotypic antibodies can be developed. Until now the direct detection of anti-idiotypic antibodies specific for HLA has not been possible. These antibodies may interfere with the currently established methods of identifying HLA specific antibody. They may also account, in part, for the dramatic changes in HLA antibody levels seen often during the course of an HLA incompatible trasnplant, for example the presence of an anti-isotypic antibody directed against HLA specific antibody may lead to a false low readout on the luminex assay. The successful isolation of a patients' own HLA specific antibody population may in turn lead to successful purification of anti-HLA idiotypic antibodies prior to transplantation.

This study shows that in principle it is possible to deplete the HLA-specific antibody pool in large volumes of human plasma. This is a promising therapeutic strategy which also allows the direct isolation of HLA-specific antibody for use in various areas of research.

Chapter 9

FACTORS DETERMINING CYTOTOXICITY OF HLA-SPECIFIC ANTI-SERA

9.1 INTRODUCTION

Complement dependant cytotoxic (CDC) crossmatch was one of the tools which was used to define the early immunological 'rules' of transplantation, importantly it was observed that the presence of donor specific antibodies (DSA) defined by CDC were associated with a higher frequency of hyperacute or acute rejection [20, 25]. Consequently for many years the presence of a positive CDC crossmatch was considered to be an absolute contraindication to transplantation. CDC does have shortcomings: it is relatively insensitive, with flow cytometry crossmatching widely available as a more sensitive crossmatch technique. In addition CDC uses rabbit serum as a source of complement and therefore does not accurately represent the normal physiological condition.

CDC was also applied to screen and identify HLA specific antibodies. More recently a range of solid phase assays (most notably ELISA and luminex microbead based assays) have been developed which use purified HLA molecules bound to the appropriate solid phase platform [176]. Beads are now the most commonly used and these assays have the additional advantages of speed (particularly in antibody characterisation) and also being able to resolve specificities which can be difficult using cell-based assays because of co-expression of HLA and linkage disequilibrium. Further advantages of bead assays are the adaptability to investigate further properties of HLA reactive antibodies- recently methods have been developed to investigate the cytotoxic potential of antibodies that bind C4d and C1q [121, 123, 177]. The C1qScreen assay (OneLambda Inc) uses a R-phycoerythrin labelled anti-human C1q antibody to detect complement activating anti-HLA antibodies.

An issue with solid phase assays is that of sensitivity, with many contrasting opinions being expressed as to the clinical relevance of many of the additional HLA

reactive antibodies defined by such assays [178-181]. According to early studies the use of the C1qScreen assay in conjunction with standard microbead assays can identify clinically relevant anti-HLA antibodies and predict AMR in cardiothoracic transplantation [123, 177]. We have observed early rejection rates of just under 50% in our HLA incompatible kidney transplant cohort thus far so identification of antibody characteristics leading to prediction of rejection are crucial [90].

In contrast, CDC relies upon the detection of antibodies bound to HLA molecules present on the surface of viable donor cells, usually lymphocytes (B and T) derived from peripheral blood, spleen, or lymph node. There are a wide array of cell surface molecules that may interfere with the CDC assay and antibody binding to any of these can lead to a false positive CDC result.

A true positive CDC result however assumes that the antibody bound to the cell surface is HLA specific and is also one of the major complement fixing immunoglobulin isotypes (IgM, IgG1 and IgG3). It is the emergence of the solid phase assays that has lead to many studies aiming to define more closely the role of immunoglobulin isotype, in particular with regard to complement fixing ability, in successful solid organ transplantation [121, 182].

IgM is the most effective immunoglobulin isotype for complement activation but is usually excluded in CDC crossmatches by using DTT such that IgG reactivity can be measured. This is primarily because of the high false positive rate observed due to the presence of non-HLA specific IgM. Of the IgG subclasses, IgG3 has the greater complement fixing activity, followed by IgG1, then IgG2, with IgG4 having the least.

As has been outlined in chapters 6 and 7, the bead assay can be further exploited to measure the relative levels of IgG subclasses with the aim to address the question; ‘What determines cytotoxicity?’ The contributing factors are likely to be: 1) Quantity of antibody, 2) Subclass / function, 3) Epitope density / distribution on the target cell, 4)

Composition of antibody specificities within the serum. Or put more simply; 'Is cytotoxicity a property of an antibody or the serum?'

9.2 MATERIALS AND METHODS

9.2.1 Patients

Initially fifty one patients who received HLA antibody incompatible (HLAi) renal allografts between Jun 2003 and October 2008 at University Hospital Coventry and Warwickshire were included in the study. All recipients underwent flow cytometry (FC) and complement dependant cytotoxic (CDC) crossmatching prior to transplant [90, 91, 116].

9.2.2 Methods

CDC crossmatching was performed using donor peripheral blood lymphocytes with rabbit serum as a source of complement, and in the presence of dithriothreitol (DTT). Acridine orange / ethidium bromide cocktail was used for cell visualisation. Where anti-human globulin (AHG) augmentation was used it was done in accordance with manufacturers instructions. Patient cohort details are given in table 9.1.

Table 9.1: Patient cohort demographics. Patients were transplanted between 2003 and 2009 at University Hospital Coventry and Warwickshire as part of the HLA antibody incompatible transplant programme.

Number	51
Male/female	18/33
Mean Age (years)	40.9
Mean duration treatment for ERF	11.5
Previous transplants	
0	19/51 (37%)
1	26/51 (51%)
2	3/51 (6%)
3	3/51 (6%)
Pre-transplant crossmatch status	
CDC+ve	17/51 (33%)
FC+ve	18/51 (35%)
Microbead +ve/FC-ve	16/51 (31%)

Pan-IgG donor specific (DSA) and third party (TPA) antibody levels were monitored daily both pre- and post-transplant on the luminex platform using the Labscreen Single Antigen bead assay (One Lambda, Canoga Park, CA).

Antibody analysis was carried out in accordance with manufacturers instructions as previously described [91, 116], goat anti-human IgG1-4 isotype specific monoclonal antibodies (Southern Biotech, Birmingham, AL) were used to determine levels of IgG subclass reactivity. The validation process for these antibodies was outlined in chapter 6. Positive control beads were supplied by the vendor and in all cases their MFI exceeded 1000 with a positive/negative control bead ration >2.0 in all cases. Antibody strength was taken as raw median fluorescence intensity (MFI), and for each crossmatch the corresponding MFI values for each donor specific antibody (DSA) were summed to give a total donor reactive MFI.

Complement binding was analysed using the C1qScreen assay (OneLambda Inc) in accordance with manufacturers instructions and given in detail in the methods chapter (chapter 2, section 2.7.6). All samples were analysed using a Luminex analyser and a positive cut-off of 500 MFI was used.

The distribution of these total MFI levels were compared between cytotoxic positive and negative cases for each IgG subclass using the Mann Whitney U-test.

9.2.3 Antibody Fractionation

A panel of 1mg sHLA columns were used to selectively fractionate HLA specific antibodies from patients from the University Hospital Coventry and Warwickshire HLAi transplant programme (see chapter 8). Patient sera was subjected to successive rounds of column absorption with specific sHLA columms in order to achieve a mono-epitope specific population, and this is outlined in figure 9.1. Antibody specificity was determined by soluble inhibition analysis and luminex single antigen microbead testing (chapter 4). Antibody preparations were buffer exchanged and concentrated using 3000MWCO spin concentrators (Sartorius Stedim, UK).

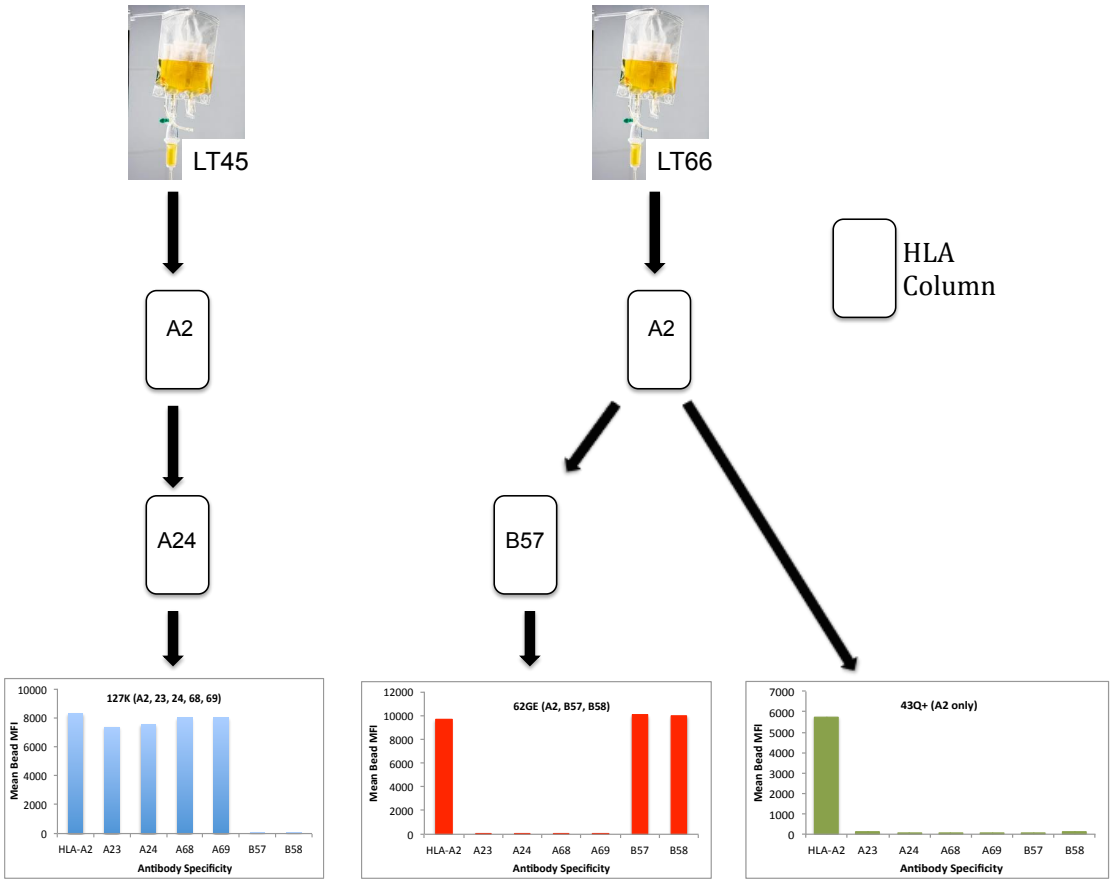


Figure 9.1: Isolation of mono-specific antibody preparations. Samples from LT45 and LT66 were re-passed through a second HLA column of selected specificity (HLA-A24 for LT45, HLA-B57 for LT66) and a single epitope specific preparation was obtained. Luminex reactivity patterns for each single specificity preparation is given.

9.3 RESULTS

9.3.1 IgG Subclass Composition and CDC Positivity

Of the 51 crossmatches 35 were CDC negative, 16 were positive. Donor specific IgG was confirmed in all cases by single antigen bead assay (One Lambda Inc, Canoga Park, CA). For all IgG subclasses, crossmatch cytotoxicity was associated with

higher donor reactive MFI levels, with median MFI values for the positive and negative crossmatch cases given in table 9.2.

Table 9.2: Analysis of median MFI levels in CDC crossmatch outcome. Median MFI values for total DSA of each IgG isotype in crossmatch positive and negative groups. Median values in each group were compared using the Mann-Whitney U test.

	Median Total DSA (MFI)		
IgG Subclass	CDC XM +ve	CDC XM -ve	*P*
IgG1	4284	854	>0.00003
IgG2	649	80	>0.0005
IgG3	210	31	>0.003
IgG4	60	10	>0.02

This data shows that the strongest association with cytotoxicity is due to the higher levels of IgG1, although the levels of all subclasses are raised in the CDC positive group (figure 9.1). However 4 of the 17 CDC positive sera lacked donor-specific IgG3 (<5 x negative control bead) whereas all contained IgG1.

This indicates that in these cells lymphocytotoxicity depended on the presence of IgG1 and that the higher levels correlate with cytotoxicity. However, figure 9.2 shows that the two highest IgG1 levels were not cytotoxic and there is a considerable overlap between the levels of CDC positive and CDC negative IgG1 antibodies. This factor alone is not a sufficient explanation of the cytotoxic property of serum.

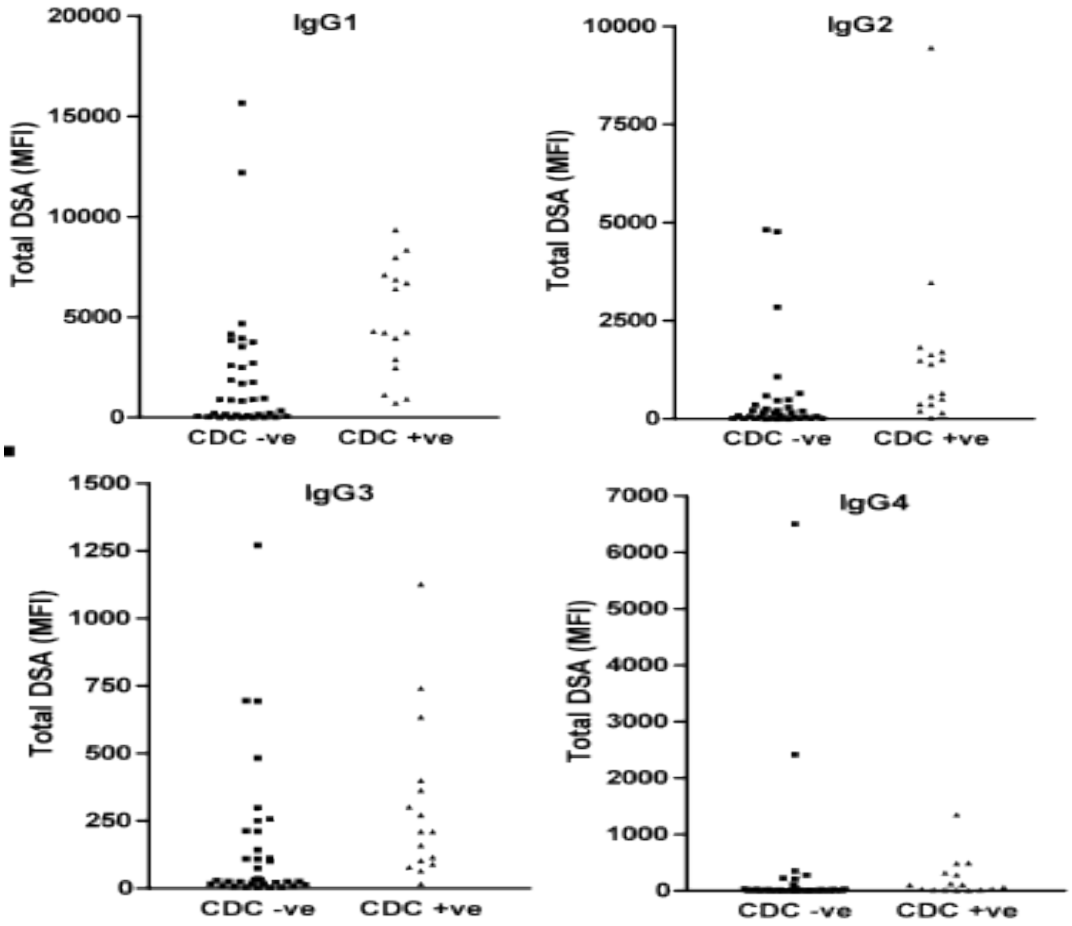


Figure 9.2: IgG subclass specific DSA levels in CDC positive and negative crossmatch cases. In general CDC positivity is associated with increased levels of IgG1, however there are notable outliers where high level IgG1 DSA does not result in a CDC positive crossmatch.

9.3.2 Correlation between the C1qScreen Assay and CDC Positivity

All 51 cases were analysed for classical complement pathway activation with the C1qScreen assay (One Lambda). Of the 16 CDC positive cases 8 had detectable C1q binding donor specific antibodies (DSA) with a mean MFI of 5219 (range: 1430-20504). In 35 CDC negative patients C1q binding antibody was negative in all but one

case where a C1q binding donor specific anti-DQ2 was detected at 853MFI, presumably below the threshold for CDC positivity (Table 9.3).

Table 9.3: Comparison of C1qScreen positivity and CDC crossmatch result. The analysis shows a significant association between CDC crossmatch positivity and detection of complement fixing antibodies using the C1qScreen assay. Test groups were compared using the Mann-Whitney U test.

	DSA		
	C1q+	C1q-	P
CDC+ (n=16)	8	8	
CDC- (n=35)	1	34	0.0002

Two cases in particular were unusual as they had high levels of donor specific IgG1 given as total DSA MFI>10000 (cases 8 and 48). There were also a small number of cases which displayed elevated IgG3 levels but were CDC negative (cases 6 and 32). Those cases with high donor specific IgG1 all tested negative for C1q binding thus confirming the earlier negative CDC crossmatch. The samples which gave the highest donor specific IgG3 levels in the absence of IgG1 were all negative for C1q binding, again confirming the earlier CDC negative. Other examples showed C1q binding HLA specific antibodies that were directed against third party antigens.

The data described here, although providing a significant correlation between CDC crossmatch outcome and C1qScreen positivity, does not provide a definitive answer to the factors defining serum cytotoxicity. There are eight CDC positive cases which still do not 'fix complement' in the C1qScreen assay. This in part shows that the lack of CDC positivity against donor lymphocytes in some cases can be explained by a lack of C1q binding. The question is why some cases of high levels of IgG1 cannot bind C1q. The explanation may be in the requirement of C1q to cross-link adjacent Fc chains, and this is explored in the next section.

9.3.3 Effect of Epitope Distribution on CDC Positivity.

It has been demonstrated in other antigen-antibody interactions that cytotoxic potential of antibody displays the hallmarks of a synergistic response, i.e the mixture of two or more antibodies against a target cell far exceeds the cytotoxic capacity of each antibody in isolation [183, 184]. Indeed, studies have shown that this synergy is greater when antibodies were directed against separate antigenic determinants on the same molecules rather than against two different determinants on the same cell [184]. With regards to HLA the mechanisms by which complement activation may potentially occur are summarised in figure 9.3.

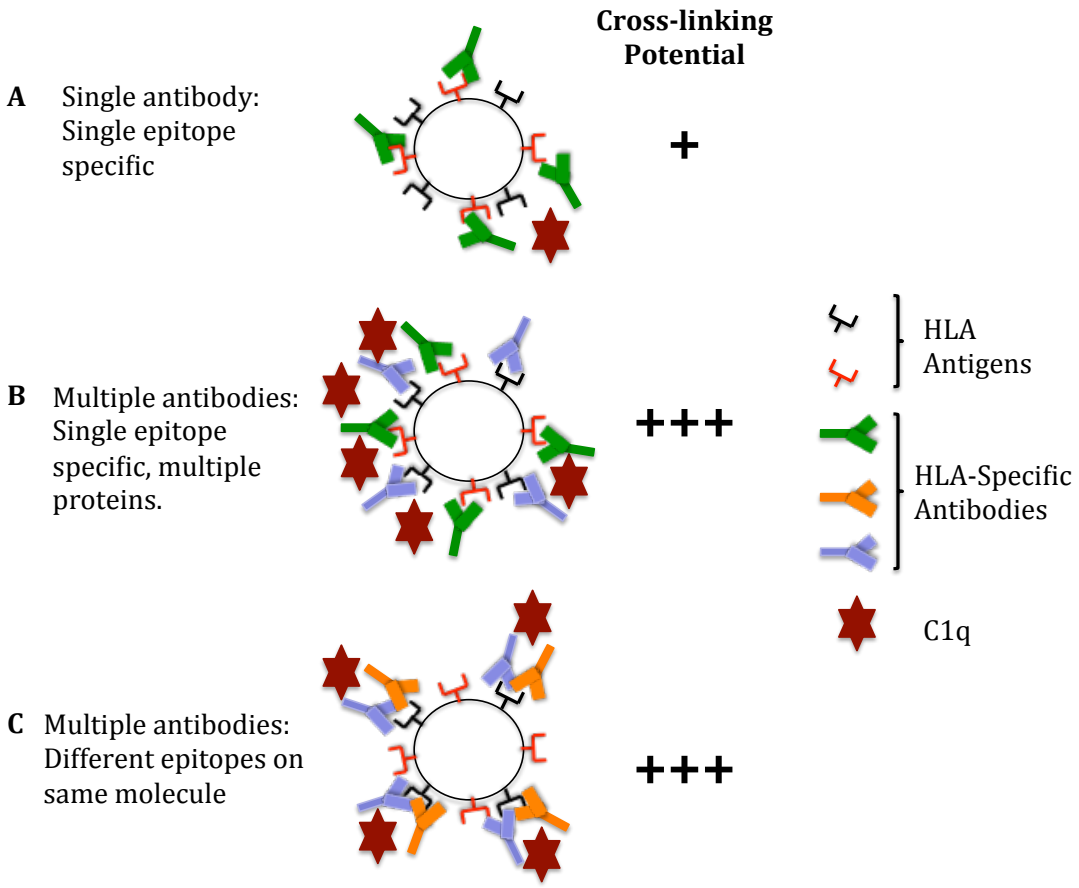


Figure 9.3: Proposed mechanisms of cytotoxicity. A) Presence of a single epitope specific antibody at a high enough level in sera to bind C1q and initiate the classical complement cascade. B) Presence of two or more antibodies recognising epitopes on different HLA molecules on the cell surface. C) Two or more antibodies specific for separate epitopes on the same HLA protein.

In the models of complement activation outlined in figure 9.3 it is important to note that: 1) There is the same amount of antibody per specificity present in each model. 2) Differences are in proximity of Fc chains and therefore C1q binding potential. 3) The prediction is that model C should show increased cytotoxicity over model B based upon the closeness of Fc proximity but this could be overruled by steric hindrance.

To investigate this, antibody eluted from patients LT45 and LT66 using the clinical scale HLA-A2 column were used (see chapter 8 for full details). For patient LT45 antibody eluted from the HLA-A2 column and which displayed reactivity specific for HLA-A2, 23, 24, 68 was re-fractionated using a HLA-A24 column, from which a preparation was obtained that contained reactivity against only the 127K epitope (HLA-A2,23,24,68,69). In the case of patient LT66 antibody eluted from the clinical scale HLA-A2 column showed reactivity against HLA-A2, B57, and B58 only with soluble inhibition data indicating the additional presence of 43Q+ epitope which is carried on HLA-A2 only (see chapter 4 for full analysis). The elutions were re-fractionated using a HLA-B57 column which was effective in producing separate mono-specific antibody preparation specific for 43Q+ and 62GE (HLA-A2, B57, B58). Specificities were confirmed using soluble inhibition assays. This process is summarised in figure 9.1.

Each mono-specific antibody preparation was then tested by microbead analysis following a serial dilution. The resulting MFI values are given in table 9.4. In addition the antibody preparations were shown to consist almost exclusively of IgG1 (table 9.5).

Table 9.4: Serial dilution of isolated HLA-specific antibody preparations. Microbead analysis of mono-epitope specific sample dilution. A pan-IgG specific labelled antibody as supplied by the manufacturer was used for detection.

	Dilution Factor					
A2 Bead Mean MFI	Neat	1 in 2	1 in 4	1 in 8	1 in 16	1 in 32
Epitope 1 (43Q+)	5750	3984	2584	2046	1180	100
Epitope 2 (62GE)	9652	7028	5293	2426	1314	402
Epitope 3 (127K)	8283	4676	2771	2205	890	416

Table 9.5: Subclass composition of fractionated antibody. Each antibody was tested with IgG subclass specific monoclonal antibody as described in chapters 6 and 7. Raw MFI values for each subclass are given.

	IgG Subclass			
A2 Bead Mean MFI	IgG1	IgG2	IgG3	IgG4
Epitope 1 (43Q+)	4544	121	8	4
Epitope 2 (62GE)	7986	88	20	2
Epitope 3 (127K)	7443	57	16	6

Next, the cytotoxic capability of each preparation individually was assessed. Each preparation was tested at a concentration range from neat down to 1 in 32 by CDC against a panel of ten lymphocytes all of which carried at least one epitope defined by the antibody preparations. The CDC was carried out both with and without the addition of AHG. The results of this analysis are summarised in table 9.6.

Table 9.6: Assessment of cytotoxic capability of single epitope binding. Without AHG augmentation the cytotoxic capacity of even high-level mono-epitope specific antibody is limited. Cytotoxic positive results are shaded red. Note, this data tests model A proposed in figure 9.3.

	Number of each epitope present on cell type			Antibody Only			Antibody + AHG		
Cell No	43Q+	62GE	127K	43Q+	62GE	127K	43Q+	62GE	127K
1	1	1	1	-	-	-	POS (1:1)	POS (1:1)	POS (1:1)
2	1	1	1	-	-	-	-	POS (1:1)	-
3	1	2	1	POS (1:1)	POS (1:1)	POS (1:2)	POS (1:2)	POS (1:4)	POS (1:2)
4	1	1	1	-	-	-	POS (1:1)	POS (1:1)	POS (1:1)
5	1	1	1	-	-	-	POS (1:1)	POS (1:1)	POS (1:1)
6	1	1	1	-	-	-	POS (1:1)	POS (1:1)	POS (1:1)
7	0	0	1	-	-	-	-	-	-
8	1	1	1	-	-	-	-	POS (1:1)	POS (1:1)
9	1	1	1	-	POS (1:1)	-	POS (1:1)	POS (1:1)	POS (1:1)
10	1	1	1	-	-	-	POS (1:1)	POS (1:1)	POS (1:1)

The next stage was to test the potential synergistic effect of multiple epitope binding upon cytotoxic capacity. Checkerboard titrations were prepared for each pair of antibodies in microtitre plates. CDC crossmatching was then performed in the absence of AHG using 21 individual cells that were once again shown to carry various numbers of the epitope sites. The class I HLA type of these cells is given in table 9.7. Each cell was also tested against each epitope specific antibody in isolation. Cells were selected to represent the presence of multiple epitopes on individual molecules and also on separate molecules expressed on the surface of the same cell.

Table 9.7: Class I HLA types of all 21 cells in the CDC checkerboard analysis.

	Class I HLA Type		
Cell	HLA-A	HLA-B	HLA-C
1	2,3	7,8	7
2	1,2	8,44	5,7
3	2,74	57,72	2,7
4	2,11	44,51	2,7
5	2,25	62,65	8,9
6	1,2	7,8	7
7	1,68	8,60	7,10
8	1,2	7,8	7
9	2,3	7,45	6,7
10	2,29	27,44	1,6
11	2,3	44,51	1,5
12	2	44,51	2,7
13	1,2	7	7
14	29,68	57,60	6,10
15	2,3	27,35	2,4
16	1,2	7,8	7
17	2,68	7,57	5,7
18	2	44	5,7
19	2,3	60,62	9,10
20	11,24	57,62	6,9
21	2,24	8,57	6,7

The determination of cytotoxicity was taken as the end point dilution at which an antibody loses its CDC reactivity when titrated into an undiluted second antibody specificity (figure 9.4). There are a number of approaches that could be taken to the determine end-point of reactivity, previous studies in other antigen-antibody interaction models suggesting this method was best suited to the format of the data here [185].

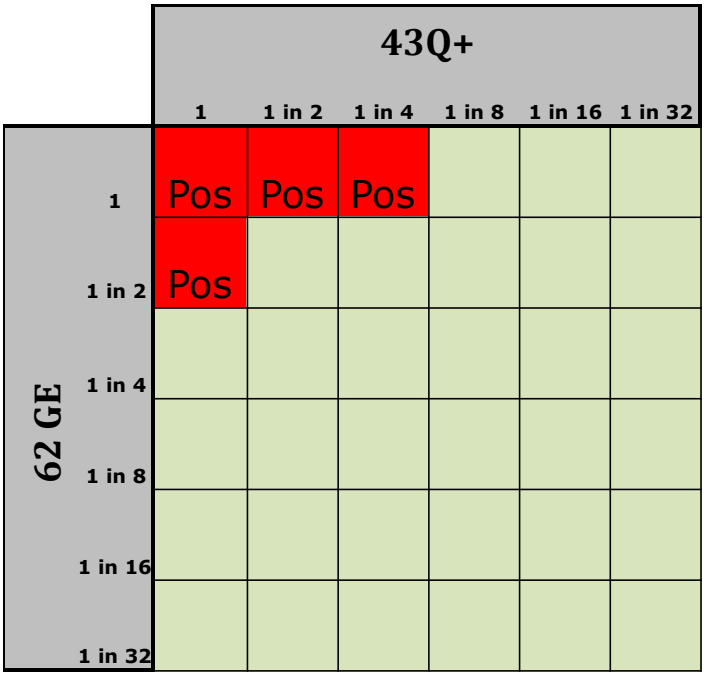


Figure 9.4: Example of epitope specific CDC checkerboard titration. In the example here the titration value of 43Q+ into 62GE would be 1 in 4. 62GE into 43Q+ is interpreted as 1 in 2.

The CDC positive threshold dilution values for each combination of epitope pair were recorded and mean values compared with the mean cytotoxic positive titre of each antibody in isolation. Figure 9.5 shows the increased cytotoxic potential of each combination of antibody pairs. Differences were analysed using Wilcoxon rank test and p values are indicated, values below 0.05 are considered statistically significant.

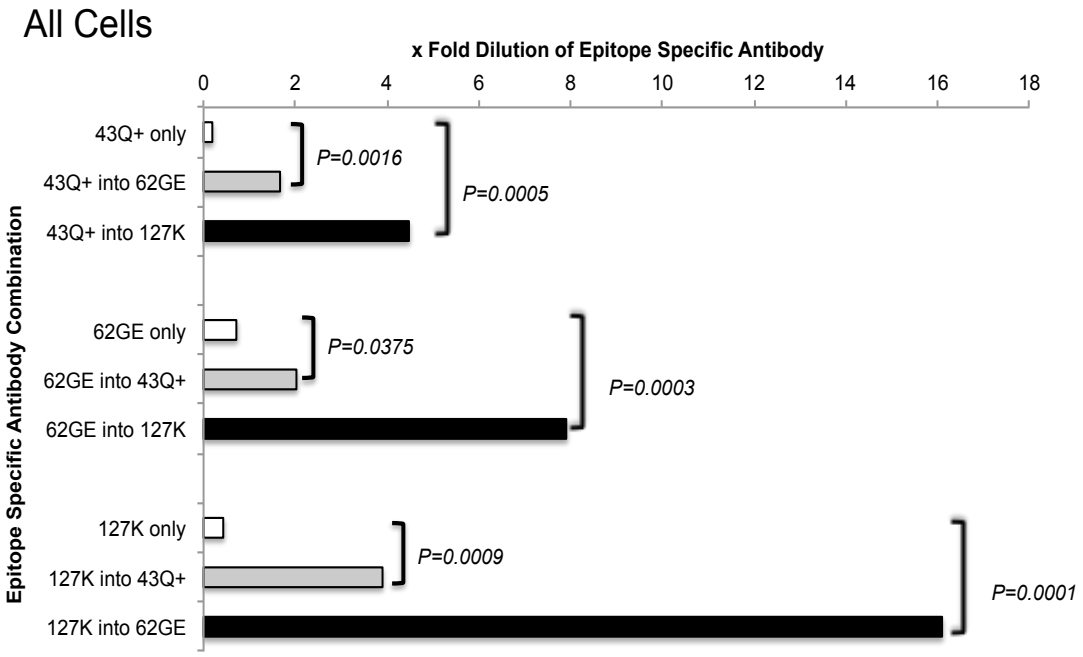


Figure 9.5: Increased cytotoxic capacity of HLA-specific antibodies due to synergy. Figure shows the mean dilution up to which CDC positivity was detected for all 21 cells tested. For all pairs of antibodies the cytotoxic capacity was significantly higher compared with cytotoxicity from each antibody tested alone.

The data in figure 9.5 show the collated data from a number of cell types therefore the CDC positivity could be due to any or all of the mechanisms highlighted in figure 9.2. Therefore the cells were divided into groups based upon epitope distribution on the cell surface in order to further explore the potential cytotoxic mechanism. Figure 9.6 shows the data from only those cells type were HLA-A2 heterozygous, meaning that all relevant epitopes were present on one HLA protein per cell- i.e this tests model C from figure 9.3.

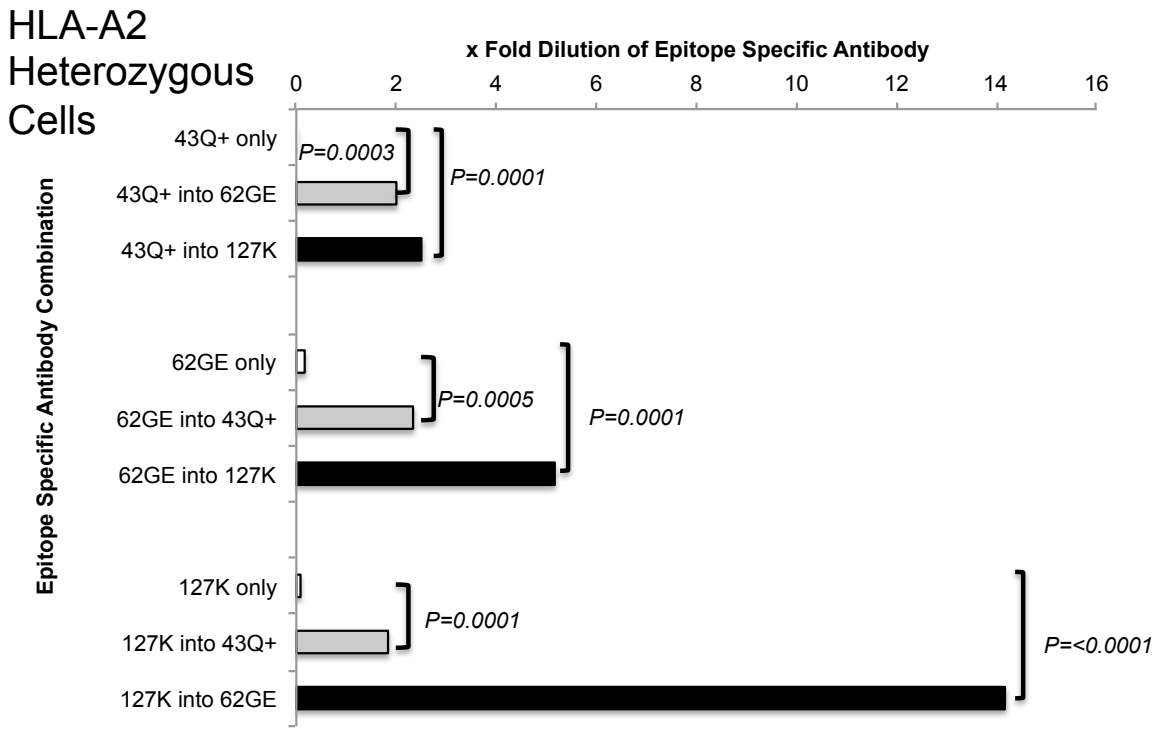


Figure 9.6: CDC synergy using HLA-A2 heterozygous target cells. Where one copy of each epitope is carried per molecule synergy is observed compared to single epitope specific antibodies in isolation.

The increased cytotoxic potential of antibodies working in synergy as shown in figure 9.6 was then applied to the cell types that were HLA-A2 homozygous. Therefore in this instance we would expect that there would be double the amount of available epitopes with which to bind and the degree of CDC reactivity would be increased. Therefore these cells can be used to again test model C, but presumably with greater epitope density. This data is shown in figure 9.7 and when compared to the dilutions at which cytotoxicity is lost in HLA-A2 heterozygous cells (figure 9.6), it can clearly be seen that CDC reactivity is increased using HLA-A2 homozygous target cells.

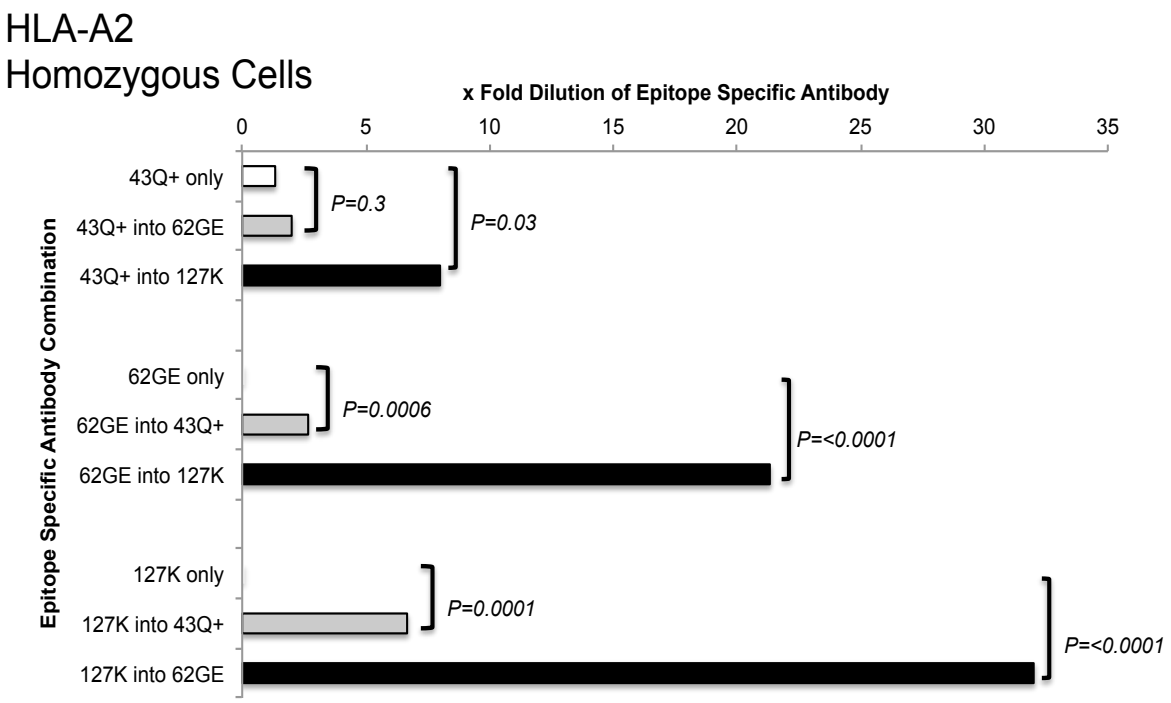


Figure 9.7: Increased CDC reactivity using HLA-A2 homozygous target cells. CDC reactivity of most paired combinations of epitope specific antibodies are enhanced compared to binding to HLA-A2 heterozygous cells.

Two cells were tested that carried the 62GE and 127K epitopes on HLA proteins other than HLA-A2. The class I HLA types of each of these cells were:

HLA-A3, 24; B57, 63; Cw6,9

HLA-A29, 68: B57, 60: Cw6,10

These cell types can be used to directly assess the cytotoxic capacity of model B, as the only epitopes represented on each cell are 62GE (by presence of HLA-B57 on each cell), and 127K (represented on HLA-A24, and A68 respectively).

The mean level of CDC reactivity of these cells is shown in figure 9.8. As would be expected it can clearly be seen that only those epitope combinations that do not include the HLA-A2 restricted 43Q+ display CDC reactivity.

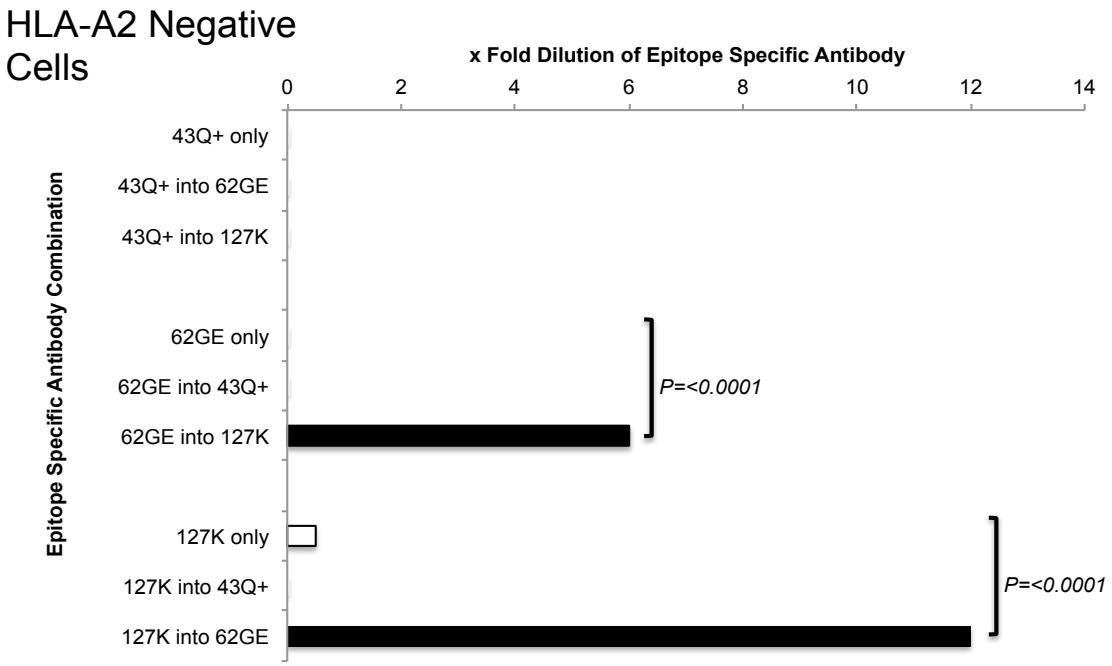


Figure 9.8: CDC reactivity against non-HLA-A2 restricted epitopes. CDC reactivity is demonstrated against the specific epitope combination that is shared between both cells. 62GE is carried by HLA-B57 in both cells and 127K is carried by HLA-A24, and A68 respectively.

In addition, a single cell used in the analysis had only 127K HLA-A68 as a potential epitope target. CDC reactivity was not achieved in this cell by any combination of antibody pairs. Again this cell represents the single epitope mechanism (model A) assumed to have poor cytotoxic potential.

This data set can be used to compare the cytotoxic potential of the proposed mechanisms of CDC reactivity outlined in figure 9.3: By separately analysing groups of

cells chosen to be representative of each scenario we can analyse the cytotoxic potential of each mechanism. Table 9.8 shows the combination of antibody and cell types required to isolate each proposed mechanism.

Table 9.8: Combinations required to analyse proposed mechanisms of CDC reactivity. The three mechanisms outlined in figure 9.3 can be analysed separately using the antibody / antigen combinations given below.

Proposed Mechanism of CDC Reactivity	Antibody Combination	Cell Type
A) Single Epitope Specific Antibody	Each Individual Epitope Specific	All A2+ve cells
B) Multiple Antibodies: Epitopes on Different Molecules	62GE / 127K Combination	A2-ve, +ve for 62GE, 127K
C) Multiple Antibodies: Epitopes on Same Molecule	All Antibody Pairs	All A2+ve cells

The potency of each combination in the CDC assay is shown in figure 9.9. The mean titre point for each antibody combination is given for each mechanism. Figure 9.9 clearly seems to suggest that the most potent mechanism for complement activation is where there are two separate epitope specific antibodies binding to their target on two separate molecules in close enough proximity to each other to cross-link C1q and initiate the classical complement pathway. However, the data set for this combination only equates to the mean of three cells tested as opposed to 12 cells for the multiple epitopes on the same molecule model.

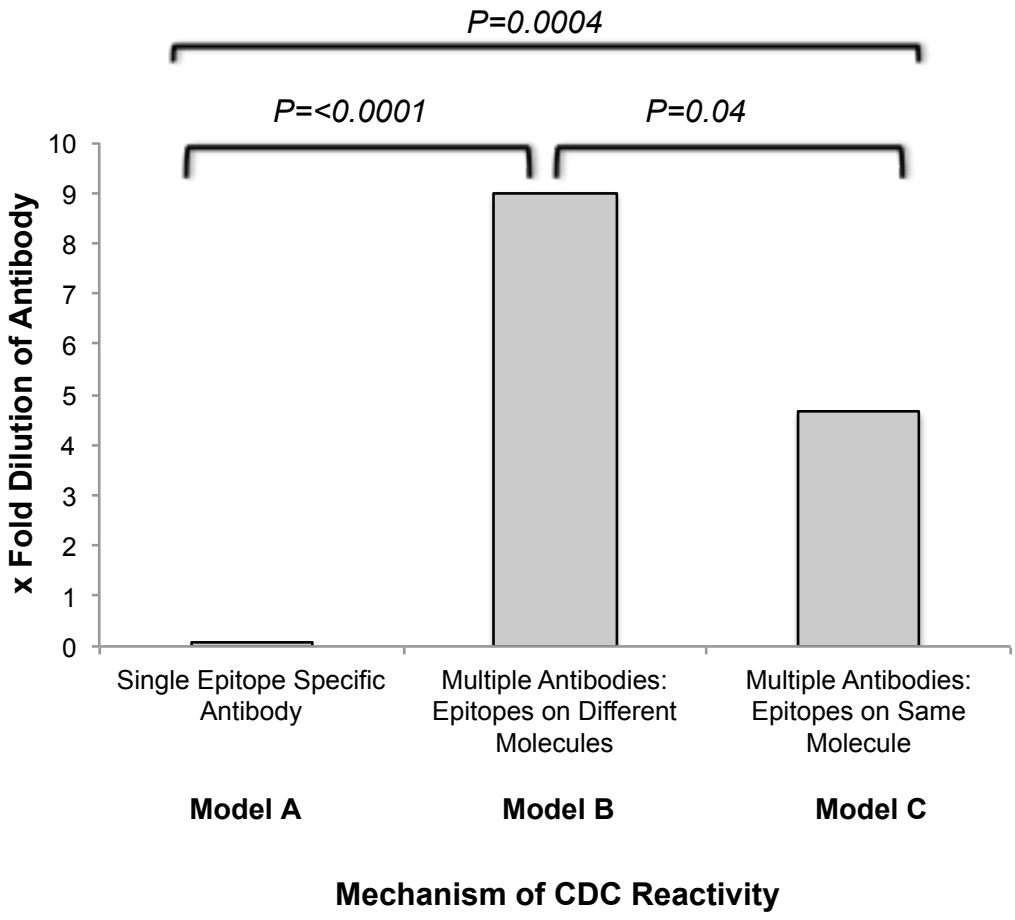
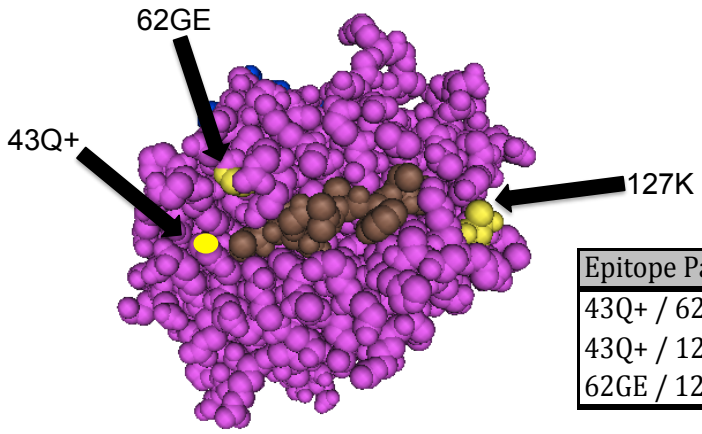


Figure 9.9: Potential CDC reactivity of the different proposed mechanisms of action. Data suggests that the binding of multiple antibodies to different epitopes carried on separate proteins on the cell surface confers the highest degree of CDC reactivity.

The data in figures 9.6 and 9.7 shows that the greatest degree of synergy occurs between the antibody pairs 43Q+/127K and 62GE/127K. The combination of 43Q+/62GE shows reduced synergistic effect, particular in the analysis of HLA-A2 homozygous cells. Figure 9.10 shows the relative positions of the epitope defining amino acids for the three antibodies. Using Cn3d Viewer software it can be seen that the

distance between 43Q+ and 62GE is approximately 9 Angstroms (Å), far closer than that between 43Q+ and 127K and 62GE and 127K (figure 9.10). This suggests that the physical spacing of epitopes is an important factor determining the cytotoxicity of antibody pairs.



Epitope Pair	Distance Apart (Å)
43Q+ / 62GE	9
43Q+ / 127K	26
62GE / 127K	24

Figure 9.10: Positions of the 43Q+, 62GE, and 127K epitopes on the HLA*02:01 molecule. The greater distance between 43Q+ and 127K and 62GE and 127K compared to between 43Q+ and 62GE appears to allow a greater degree of cytotoxicity.

By separating the data set for multiple epitopes recognised on the same molecule into the separate antibody pairs is possible to investigate this further. Figure 9.11 shows the result of this analysis.

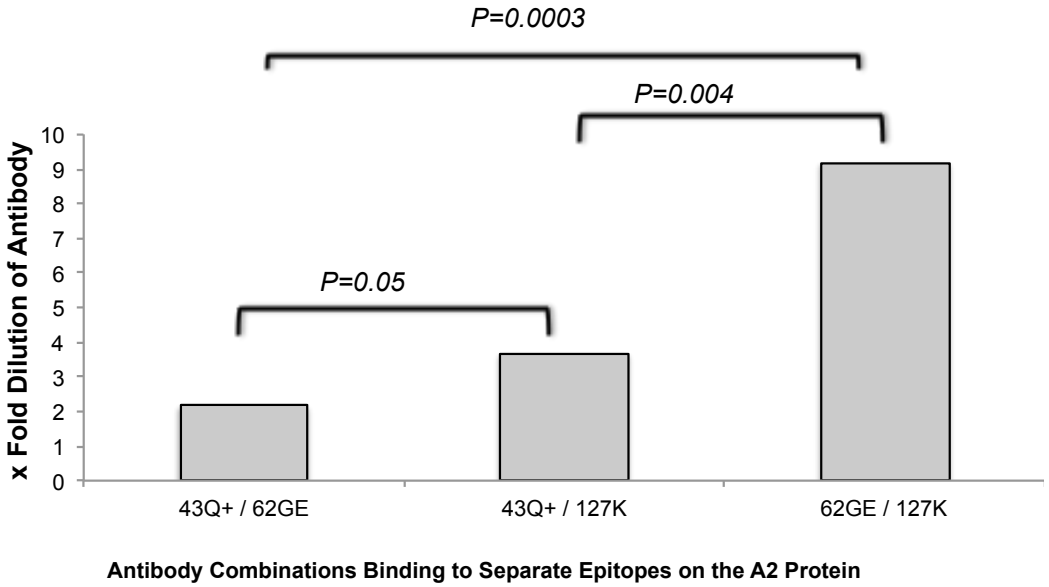


Figure 9.11: Effect of epitope spacing on CDC reactivity. The higher degree of spatial separation on the HLA-A2 protein of the 43Q+ / 127K, and 62GE / 127K combinations leads to greater CDC reactivity.

The most potent combination of antibodies is clearly 62GE and 127K with a reactivity strength that matches that of the separate epitopes on distinct protein model data shown in figure 9.9. The observation that 43Q+ / 127K also displays enhanced synergy, albeit to a lesser extent, than the 43Q+ / 62GE combination also supports the theory that steric hindrance may be a factor in defining serum cytotoxicity. The microbead analysis of the single epitope specific antibody preparation gave a dose response curve consistent with the dilution of the antibody level (figure 9.12A). However, when the epitope specific pairs were titrated into each other as per the CDC checkerboard analysis and then tested with microbead analysis, it can clearly be seen that the 43Q+ / 62GE combination displays the features of the high dose hook effect

(HDHE) as outlined in detail in chapter 3 (figure 9.12B). The other antibody combinations do not show this effect, suggesting a degree of steric hindrance within the luminex assay. In addition as these samples were purified IgG preparations we can discount the potential role of IgM interfering with the binding within the luminex assay as has previously been reported [119].

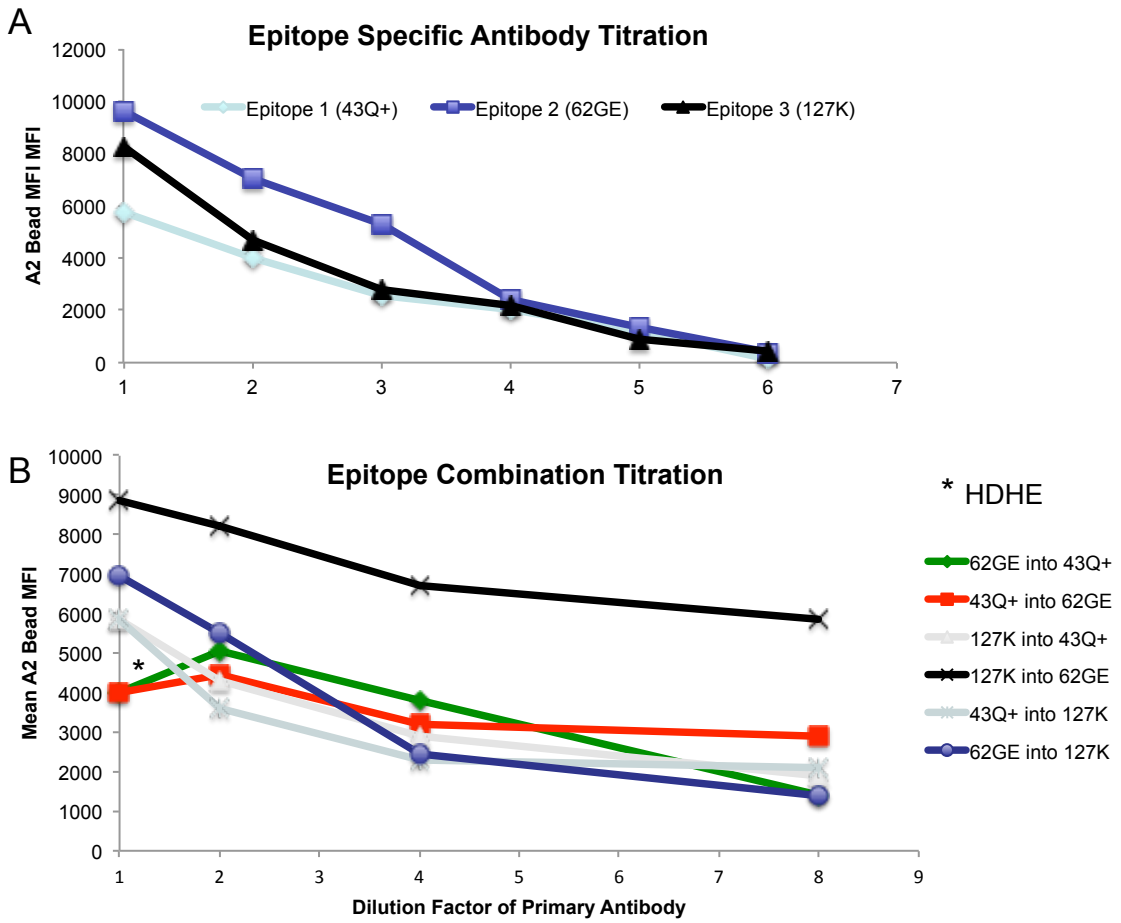


Figure 9.12: Competition for epitopes leading to the detection of the high dose hook effect. A) Titration of single epitope specific antibody samples shows a fall in MFI with increasing dilution of antibody as expected. B) Titration of epitope pairs reveals the presence of the high dose hook effect in the 43Q+ / 62GE combination which bind to separate epitopes very closely located on the surface of the HLA-A2 protein.

9.4 DISCUSSION

9.4.1 IgG Subclass Level and Distribution

One of the major dilemmas faced by the modern tissue typing laboratory is how to interpret the antibody analysis data provided by the highly specific solid phase assays that are now commercially available. The opinion of the transplant community is split on how to define those antibodies that are clinically relevant [178-181, 186].

One area that seems to provide more clarity takes us back to the early days of transplantation and that is the presence of a CDC positive crossmatch and the associated increased risk to successful transplantation. Indeed recent reviews call for a risk stratification algorithm for sensitised patients that places the CDC positive specificities at the heart of the organ allocation process [14].

But what defines a CDC positive serum? CDC positivity could be due to either the total amount of donor specific immunoglobulin present in a serum, the relative proportion of the different complement fixing isotypes (IgM, IgG1,IgG3), or a combination of both factors. Of the IgG subclasses, IgG3 has the greater complement fixing activity, followed by IgG1, then IgG2, with IgG4 having the least.

Of these factors this study clearly shows that the strongest association with serum cytotoxicity is with increased levels of circulating donor-specific IgG1 *($p>0.00003$).* There is also a significant increase in the raw MFI values of donor specific IgG2, IgG3 and IgG4. It must be noted that the majority of raw MFI values obtained for IgG3 and IgG4 in particular are clustered around the region which would be deemed negative by the laboratory. The apparent contribution of each HLA specific IgG subclass to the overall measure of cytotoxicity closely follows the proportions with which each subclass is distributed in the general circulation (IgG1 9mg/ml, IgG2

3mg/ml, IgG3 1mg/ml, IgG4 0.5 mg/ml). Furthermore, the importance of IgG1 in defining cytotoxicity is supported by the observation that 4 of the 17 CDC positive sera completely lacked any donor specific IgG3 (MFI value <5x negative control bead), and all 16 CDC positive sera contain donor specific IgG1.

9.4.2 C1qScreen Assay and CDC Crossmatch

However, this data set provided a number of outliers. For example two patients had donor specific IgG1 levels over 10,000MFI but were CDC crossmatch negative. In view of this all samples were tested with the C1qScreen assay (OneLambda Inc) which detects HLA specific antibody with the ability to bind C1q and therefore initiate the complement cascade via the classical pathway [123]. No C1q binding was observed for the DSA in these patients thus confirming the crossmatch results. This observation may suggest that IgG1 does not activate the classical complement pathway in all instances. Variation in Fab region binding would not appear to be the reason as specific binding has been observed in both the bead and flow cytometry assays, similarly antibody avidity effects are unlikely. This would suggest that variation in some part of the Fc region may account for the incomplete association between IgG1 and complement fixation. In addition those patients with the highest levels of IgG3 were all negative with the C1q assay although the highest MFI in this group was only 694. Eight of sixteen CDC positive samples showed no detectable C1q binding antibodies specific for the donor. This may suggest that the C1q assay does not represent the in vitro mechanism of classical pathway complement activation. Biologically, the spacing of the antibody-antigen complexes is crucial to effective C1q cross-linking. In the *in vitro* microbead assay the distribution of the antigen on the surface of the bead is notoriously

variable within bead batches. Thus a reduced surface expression of HLA antigen may not permit the proximity of antibody binding to be sufficient to crosslink C1q.

One case that was CDC negative gave an anti-HLA DQ2 C1q fixing antibody with an MFI of 853. The CDC result was confirmed negative in the presence of DTT. This patient provided a weakly positive flow cytometry crossmatch, suggesting that with an MFI of only 853 this level of antibody may be below the titre required for CDC positivity. Alternatively the level of HLA-DQ expression on donor derived B lymphocytes may be insufficient to enable stable crosslinking of antibody and subsequent complement activation.

9.4.3 CDC Crossmatch Predicted by Epitope Distribution

The ability to isolate HLA-specific antibodies from human plasma using HLA protein columns provided a unique tool to explore in detail the factors which contribute towards CDC positivity. This has been achieved in other antigenic systems but has not, to our knowledge, been explored on the HLA setting. All three of the potential mechanisms outlined in figure 9.2 were shown to have at least some degree of CDC reactivity when tested using epitope specific purified antibodies. It has been clearly shown that when there is a single epitope specific antibody present, regardless of the level of that antibody it is highly unlikely that any reactivity within the CDC assay will be observed. The likely reason for this would be that the binding only occurs in one place on the HLA molecule, therefore to activate complement via the classical pathway the co-localisation of two or more separate HLA molecules on the cell surface would be required (figure 9.2A). The distribution of HLA on the cell membrane may be such that the antibodies would not be in close enough proximity to cross-link C1q.

Where there is another epitope specific antibody in addition it is clear that the cytotoxic potential of a serum would be greatly enhanced. Figure 9.9 shows the binding of two antibodies specific for two separate HLA molecules present on the surface of the leads to enhanced cytotoxic potential via the mechanism suggested as model B in figure 9.2. Similarly, the binding of two antibodies to separate epitopes on the same molecule leads to greatly enhanced cytotoxicity. This has been reported before in systems other than HLA [184], however this study has gone a stage further and demonstrates that this synergy can be inhibited if the epitope sites are situated in too close proximity on the cell surface (figure 9.11).

In addition, the spacing of epitopes on the molecule has a significant impact upon the ability of antibodies to bind to HLA-coated microspheres (figure 9.12). The presence of multiple antibodies within a serum that are specific for epitopes found in close proximity on the molecule may introduce a degree of steric hindrance, which has been demonstrated here to result in the high dose hook effect. The consequences of this for patient antibody monitoring and subsequent clinical intervention are discussed in detail in chapter 3, but this added layer of complexity further confounds attempts to accurately quantify the HLA-specific antibody response using microbead assays.

The earlier analysis of C1qScreen data in CDC positive cases showed that a CDC positive crossmatch could be correlated to a detectable C1q binding DSA in only 8/16 cases. These findings could help to explain why there is not 100% concordance between CDC crossmatch results and the C1qScreen assay. The manufacturer's recommend that single antigen HLA beads are used to detect C1q binding antibody. However, classical pathway complement activation requires the proximity of at least two antibody molecules in order to corss-link C1q. The use of the single antigen microbead assay immediately removes the potential to cross-link C1q by two antibodies recognising epitopes on different molecules as only one protein is expressed on the

bead. This study suggest that this mechanism is as important as antibodies binding to multiple epitopes on the same molecule, therefore we can speculate from this that the 50% concordance between CDC and C1qScreen is a direct consequence of having this route of CDC activation omitted. Furthermore, if we consider some of the individual patient data provided in table 9.4, we have evidence to support this hypothesis. Cases 2 and 33 both have detectable IgG1 and have a donor-specific C1q binding antibody of 12,000 and 6,500 MFI respectively. In both of these cases the donor specific antibody is broadly defined as being directed against HLA-A2, however, as has been demonstrated experimentally in chapter 4 by epitope specific soluble inhibition, and in chapter 8 by HLA column specific elution, HLA-A2 'specific' antibodies are almost always a compound of two or more antibodies recognising separate epitope carried by the HLA-A2 protein. This particular combination should be detected in the C1qScreen assay as multiple antibodies are able to bind to each HLA-A2 molecule present. Conversely, patients 8 and 48 also displays high levels of donor specific IgG1. Patient 8 had a HLA-B63 specific DSA at 15,660 MFI and for patient 048 a HLA-A3 specific DSA at 12203 MFI. In both of these cases the antibody reactivity measured by luminex is far more likely to be caused by a single epitope specificity and therefore be unable to complex C1q within the C1qScreen assay. In both cases this was confirmed by soluble inhibition assays using the requisite soluble HLA specificities (figure 9.13).

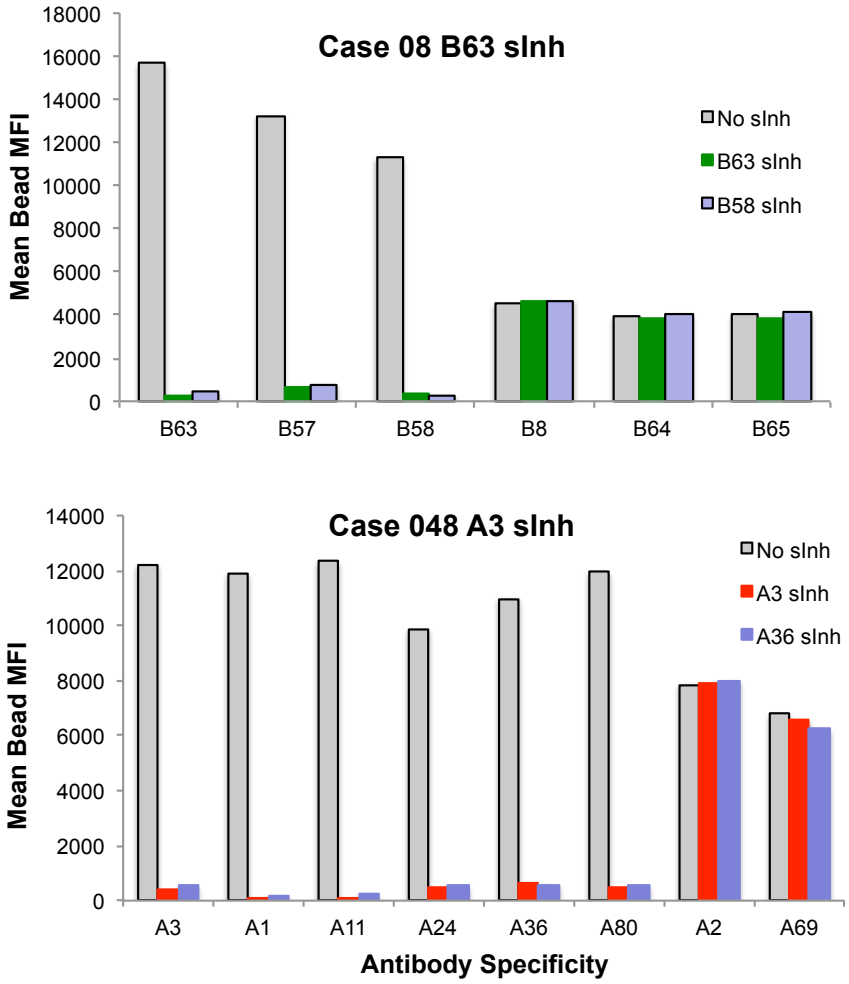


Figure 9.13: Soluble inhibition (sInh) analysis to confirm that DSA binding is due to single epitope recognition. In case 8 the DSA is specific for the 65R+/66N epitope shared with HLA-B57 and B58. This was confirmed by sInh using soluble HLA-B63 and B58. In case 48 the HLA-A3 DSA recognised the 144K+ epitope shared by HLA-A1,11,24,36,80. Entire reactivity was depleted by both HLA-A3 and A36 soluble inhibition. For both cases selected third party antibody specificites are shown.

From this we can conclude that the clinical utility of the C1Screen assay is limited whilst the assay is used with single antigen specific microbeads. A more suitable

alternative might be to use the C1qScreen assay with phenotype beads (microbeads that carry intact HLA haplotype) such that all potential mechanisms of C1q binding can be incorporated. Although it is important to point out that cells and beads also differ fundamentally in that HLA present on cells has the ability to move within the cell membrane, whereas microbead coupled HLA is immobile.

In summary, the factors which determine the cytotoxicity of HLA-specific anti-sera have been investigated and the IgG subclass composition and level are significant factors. It has been demonstrated that HLA-specific antibodies act in a synergistic manner at the cell surface, therefore the cytotoxic effects of the mixtures of antibodies far exceeded the additive effects of their component antibodies in isolation. Synergy was comparable between binding to multiple proteins and to multiple epitopes on the same protein, but crucially cytotoxicity was enhanced when binding to epitopes on the same molecule occurred between two spacially separated sites.

Chapter 10

CASE STUDY:

HLA INCOMPATIBLE COMBINED LIVER-KIDNEY TRANSPLANTATION: DYNAMICS OF ANTIBODY MODULATION.

10.1 BACKGROUND

Preformed donor HLA-specific antibodies (DSA) present at the time of kidney transplantation can cause hyperacute or accelerated rejection of allografts and are generally accepted as contraindications to conventional transplantation. Recent developments have mitigated this risk by antibody removal or inhibition using plasma exchange, high dose intravenous immunoglobulin, or complement inhibitors [105, 129]. However, these techniques require preparation pre-transplantation and are therefore currently best suited to elective, live donor transplantation. Unfortunately, not all patients have available live donors, and for highly sensitised individuals the chance of finding a suitably matched organ is generally low. Important clinical series from Olausson and colleagues report good outcomes from combined liver-kidney transplantation in the deceased-donor setting, the presumed mechanism of which is antibody absorption onto the liver which is implanted before the kidney, and is itself resistant to immediate antibody mediated rejection [187, 188]. Despite such reports, data is lacking to describe the dynamics of HLA-specific antibodies following transplantation, and how these correlate with outcome. Herein we describe a case where combined liver-kidney transplantation was performed in the presence of high level, cytotoxic, donor specific anti-HLA antibodies. In particular, we describe detailed characterisation and post-transplant dynamics of these antibodies, specifically regarding epitope reactivity, which may help explain the outcomes of these potentially high-risk procedures.

10.2 DESCRIPTION OF THE CASE

A 43-year-old lady with autosomal dominant polycystic kidney disease presented for combined liver and kidney transplantation. Two years prior to transplantation, she complained of increasing weight loss, anorexia, early satiety and right-sided hypochondrial discomfort. Imaging revealed liver enlargement causing compression of upper abdominal viscera. Despite conservative measures, weight loss continued and she was listed for liver transplantation to alleviate the mass effect of her native liver. A gradual decline in renal function over time was observed, and when her estimated glomerular filtration rate (eGFR) fell below 25ml/min kidney transplantation work-up was initiated. As part of this, antibody reactivity against multiple class I HLAs (negative against any class II) was identified by single antigen bead assay (LSA104, One Lambda Inc, Canoga Park, CA), with a calculated reaction frequency of 99%. She had not received blood products or a transplant, and the assumed source of sensitisation was four pregnancies.

10.3 TRANSPLANTATION- CLINICAL COURSE

Transplantation proceeded 18 months after listing. Immunosuppression consisted of anti-CD25 monoclonal antibody induction, tacrolimus (0.3mg/kg/day), mycophenolate mofetil (2g/day) and methylprednisolone 500mg at operation followed by oral prednisolone 20mg enterally then tapering. Intravenous immunoglobulin (IVIG) was administered (0.5g/kg/day) for the first five post operative days. The surgical procedures were uneventful and immediate liver and kidney graft function established. By post-operative day 5, her serum creatinine had fallen to 108μmol/L. However,

between days seven and ten the serum creatinine rose progressively to 197μmol/L. A kidney biopsy undertaken on day nine displayed acute tubular injury with vacuolation, diffuse C4d staining, but no inflammatory infiltrate in interstitium, large vessels, peri-tubular capillaries or glomerulus (Figure 1b,c). The tacrolimus level on the day of biopsy was 15ng/ml (high performance liquid chromatography/tandem mass spectrometry). A clinical diagnosis of tacrolimus toxicity was made, and the tacrolimus dose reduced resulting in a tacrolimus level of 8ng/ml three days later. Renal function subsequently improved (day 13 creatinine: 145μmol/L; day 16 creatinine: 120μmol/L). No adjunctive escalation in immunosuppression was undertaken; her subsequent clinical course was uneventful with stable renal function 40 months post-transplantation (serum creatinine:126μmol/L; eGFR:47ml/min; urine albumin:creatinine ratio:14.2mg/mmol).

10.4 POST-TRANSPLANT ANTIBODY DYNAMICS

The recipient and donor HLA types were A23,30; B44,53; Cw6; DR11, 15; DQ 6,7; and A2,3; B7,60; Cw7,10; DR 8,15; DQ4,6; respectively. The patient's immediate pretransplant serum was reactive against all class I HLA mismatches except A3 giving a cumulative donor-specific reactivity corresponding to an MFI of 55,000 (sum of all DSA bead values). Forty minutes following liver arterial anastomosis (just prior to kidney implantation) a further serum sample was taken followed by daily samples for two weeks, then at weekly intervals. Changes in MFI values for each bead carrying HLA corresponding to donor mismatches are shown in Figure 10.1a, and the following key points noted: 1] The sample taken 40 minutes following liver transplantation showed a dramatic reduction of all detectable HLA antibody specificities (including

those against non-donor antigens). Thus all the patient's HLA-specific antibodies were absorbed by HLA epitopes on the donor liver. 2] Retrospective cytotoxic crossmatches (complement-dependent, CDC, without AHG augmentation) showed a strong reaction (8 score) with the pretransplant sample, and a negative reaction with the 40 minute sample. 3] On day five reactivity against HLA Cw7 returned (MFI: 2,150), and the remaining DSAs reappeared on day six. 4] Between days nine and ten all DSAs appeared to return to their pretransplant levels after which all DSAs spontaneously fall, and all except those for Cw7 return to baseline 35 days post-transplantation. 5] Finally, the specificities of the pre- and post-transplant antibodies are the same and differ only quantitatively.

Examination of the pre- and the peak post-transplant levels for each DSA (Figure 10.2a) shows that two DSAs rise above pretransplant levels (A2 and Cw7), B7 reactivity is unchanged, while reactivity against both B60 and Cw10 is greatly reduced. Assuming antibody levels measured in the post-transplant samples represents the difference between adsorption and synthesis, this shows it took at least five days for antibody resynthesis to overcome adsorption onto donor tissue. This therefore suggests that the relative rates of adsorption and synthesis vary for the different DSAs. For example, after day five the synthesis of antibodies binding to Cw7 greatly exceeds their adsorption rate (rapidly rising serum level) but less so for Cw10-binding antibodies. Assuming there is equivalent expression of these two antigens then the difference is likely related to their different rates of synthesis. However, this assumption is partially inaccurate because HLA specific antibodies are composed of epitope-specific antibodies, and such epitopes can be shared between different HLA allotypes and isotypes. Thus antibody reactivity against Cw7 and Cw10 may be due to a shared

epitope and therefore the same antibody. Indeed this seems to be partly the case, as demonstrated in the following analysis:

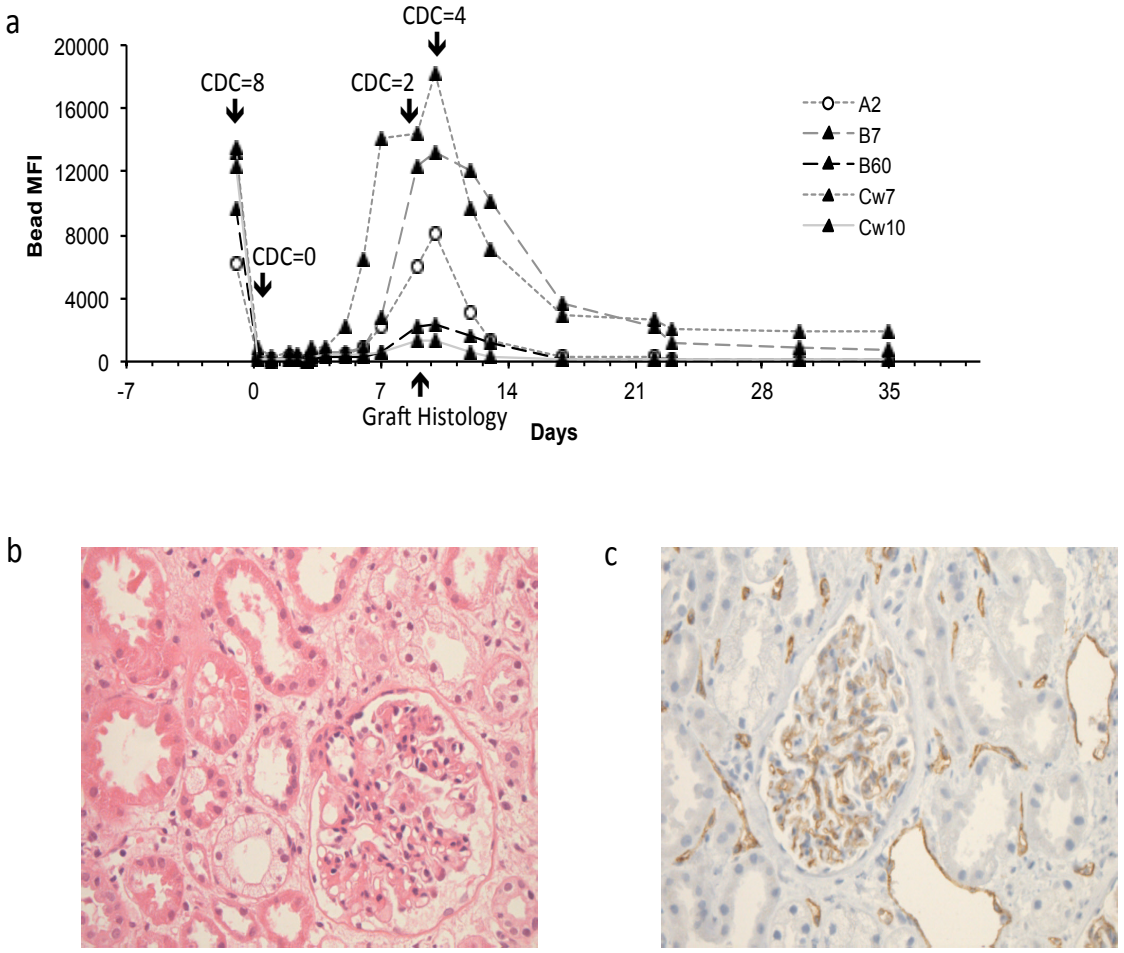


Figure 10.1: Changes in antibody levels and changes in histology over the early post-transplant period. a) Changes in donor reactive bead MFI values through the early pre- and post-transplant period; transplant on day 0. b,c) Kidney graft histology at day 9 post transplantation. b) H&E staining showing vacuolation of occasional proximal tubules without cellular infiltrate; c) C4d immunohistochemistry showed diffuse linear staining of glomerular and peritubular capillaries.

Epitope specificities of the recipient's antibodies were determined using the Matchmaker programme, specifically designed for this purpose and freely available [81, 82, 146, 189]. Epitopes are described in accordance with previously published notations [80, 147] and an estimate of epitope reactivity in the recipient's sera is given in Table 10.1a, which shows which antigens carry the respective epitopes. Note that one epitope, 80N (corresponding to the serological type Bw6), is present on four of the mismatched antigens, and epitopes 253Q and [163E+166E] are each carried by two separate mismatched antigens. Conversely, HLA A2 and B60 reactivities are likely due to two different epitope-specific antibodies in each case, and both B7 and Cw7 reactivities are due to three epitope-specific antibodies. As Cw10 reactivity seems to be due only to antibody specific for 80N the attenuated post-transplant response to this antigen might more accurately be described as an attenuated response to 80N. As four mismatched antigens carry 80N it could be the greater representation of this epitope increasing the adsorption rate of the corresponding antibody. To track this and the other epitope-specific antibodies, selected specific beads from the bead assay were chosen that carry the minimum number of each of the epitopes shown in Table 10.1b, and we analysed these rather than the beads matching the donor HLA. The selected beads and results of this analysis are shown in Figure 10.2b. Four beads were selected that each carried a single epitope recognised by the patient's sera, although there were no beads to resolve 70Q from 80N, 253Q from 267Q or 142T from 253Q.

a. HLA mismatch	Epitope specificity of donor reactive antibody					
	142T	253Q	80N	70Q*	267Q	[163E+166E]
A2	+	+				
A3						
B7			+	+		+
B60			+			+
Cw7		+	+		+	
Cw10			+			

b. Tracker antigens						
A69	+	+				
A25		+				
B47						+
B42			+	+		
B8			+			
Cw17		+			+	

Table 10.1: Summary of donor specific reactivity. Reactive epitopes are shown alongside the HLA mismatched donor antigen (a) and the third party antigens (b) on which they are expressed. *The full description of this epitope is given as [41A+46E+67Y/43P+46E+67Y/43P+46E+70Q]/43P+69A+70Q/43P+70Q+76E/[46E+65Q+67Y/46E+65Q+70Q] in El-Awar et al (10); this has been abbreviated to 70Q for ease of use.

The changes in epitope-specific antibodies are expressed as percent of starting level to normalise the data and take account of variation in antigen density between beads, and pre- and peak post-transplant levels compared to show the maximal response of each antibody (Figure 10.2b). The lowest response at peak was seen for antibodies against epitopes 253Q and 80N, at 10% and 20% of their pretransplant levels. In contrast the peak level of [163E+166E] specific antibody was 440% of the pretransplant level. The level of antibody against 70Q appears to be similar for pretransplant and peak (80%) although this is confounded by 80N. The response against 267Q could not be directly assessed as the beads carrying this also carried confounding epitopes, but using a Cw17 bead (253Q and 267Q) bead a 180% change from pretransplant to peak level was observed and, as we know that the antibody against 253Q was greatly reduced, it is clear that the increase against this Cw17 bead is due to reactivity against 267Q and that the change in this antibody is likely to be underestimated. Similarly the response against 142T is likely to be greater than the 150% when tracked with the A29 (142T and 253Q) response.

When approached from the perspective of epitope-reactivity, dramatic responses are evident which are not apparent when looking at whole antigen-specific responses. The response measured against an antigen gives a net change: thus the peak level of antibody against HLA B7 was similar to the pretransplant level. This is because the B7 reactivity is due to antibodies against 70Q (little change), 80N (reduction) and 163E+166E (increase). Indeed if the MFIs of the three epitope-specific reactions are summed, the result is 18,700 for the pretransplant sample and 15,100 for the peak post-transplant sample. A rebound to pretransplant levels suggests a null humoral response to the graft whereas an increase indicates an active, memory response.

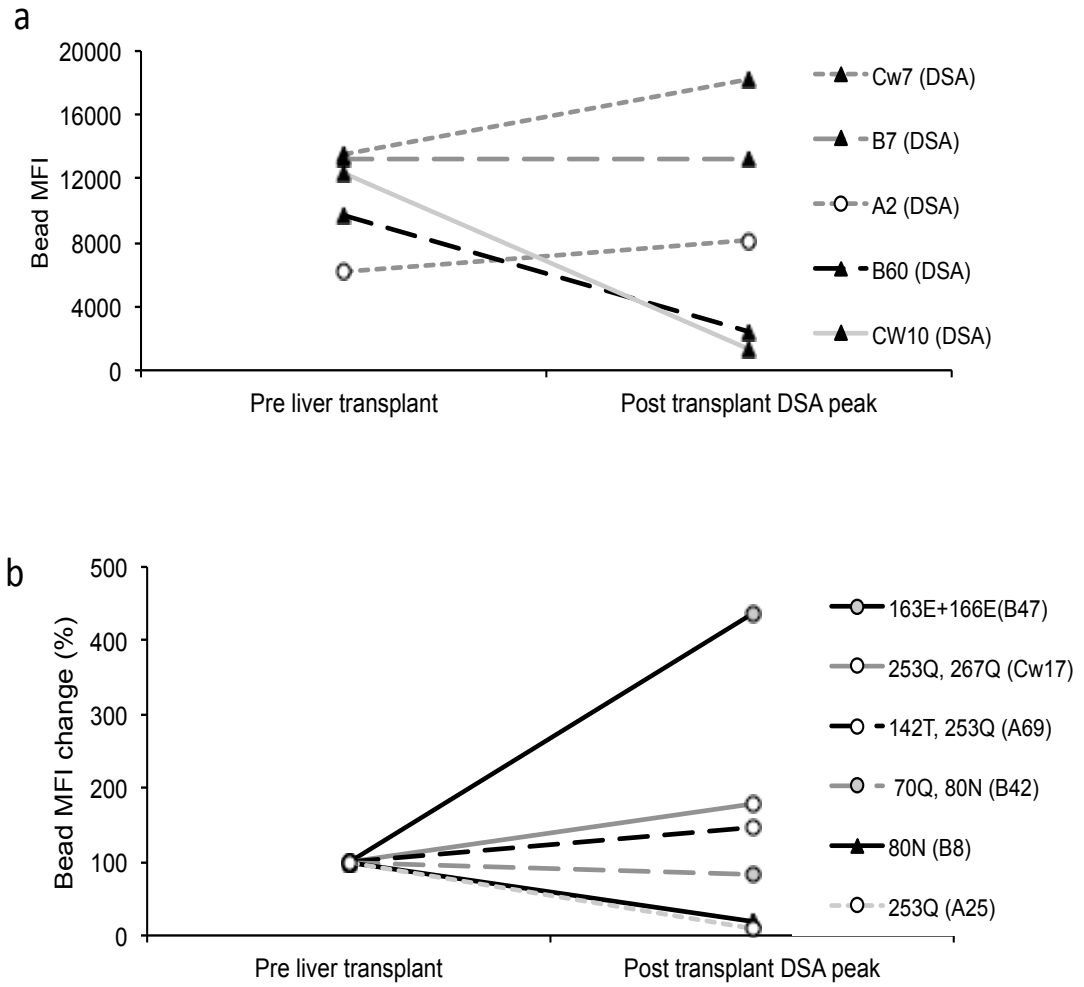


Figure 10.2: Changed in antibody levels assessed using the bead specificities and refined epitope specificities. a) Overall change in donor reactivity from pre-transplant to post-transplant DSA peak for each donor-specific antibody. b) Net change in donor reactivity from pre-transplant to post-transplant peak DSA for each epitope specific donor reactive antibody. Surrogate antigens were used to analyse epitope-specific reactivity.

Although overall the pretransplant and peak post-transplant levels were broadly similar, the reaction pattern of the HLA-specific antibodies changed dramatically with these changes coinciding with changes in serum cytotoxicity. The pretransplant serum

was strongly cytotoxic (killing score 8), but at postransplant days 9 and 10 when the overall antibody levels had reached or exceeded the pretransplant levels, cytotoxicity was noticeably lower with killing scores of 2 and 4 respectively (shown in Figure 10.1a). At this timepoint, serological reactivity was dominated by antibodies against 142T (A2), [163E+166E] (B7 and B60), and 267Q (Cw7) while antibodies against 80N fell dramatically. Perhaps these antibodies intrinsically differ in their pathogenicity or perhaps certain combinations of antibodies are required for optimum cytotoxicity as suggested in chapter 9. Serum creatinine did rise with the rising donor-specific antibodies (and, interestingly, C4d staining on biopsy) but tacrolimus levels were above target, and tacrolimus nephrotoxicity implicated, with an improvement in graft function following tacrolimus dose reduction and subsequent stability over considerable follow-up. High levels of early antibody resynthesis were therefore seemingly insufficient to cause rejection. Although it is tempting to speculate that this is a protective effect of the liver, this phenomenon has also been described in more conventional HLA antibody incompatible kidney transplantation [90].

The final phenomenon revealed by frequent antibody testing during the first few post-transplant weeks is the rapid rise followed by a seemingly spontaneous fall in antibody levels. No treatment such as IVIG, rituximab or bortezomib, was used to modulate the resynthesis of the DSA following its reappearance, yet by post-operative day 35 most of the antibodies had all but disappeared. The cause of this modulation is unknown but again this is not unique to combined liver-kidney transplantation, having been described in antibody-incompatible transplantation following extracorporeal antibody removal [91].

In summary, this case confirms the utility of simultaneous liver transplantation in allowing successful kidney transplantation in the face of preformed, high level DSA,

which would normally be associated with hyperacute rejection and kidney graft failure. Antibody characterisation in terms of epitope specificity is more accurate and informative than antibodies described as “antigen-specific” and a method is suggested here for identifying and tracking these antibodies - i.e. follow the epitope reactions and not the antigen reactions. This will give better insight into the behaviour and pathogenicity of HLA-specific sera. In the case presented here this approach has revealed some novel features of the post transplant antibody response in a sensitised recipient. These illustrate three phenomena that challenge current dogmas - an early resynthesis of DSA does not necessarily cause antibody-mediated rejection, high levels of DSA can spontaneously modulate, and measurement of antibodies in terms of solely antigen specificity can give misleading results.

Chapter 11

SUMMARY AND FUTURE DIRECTIONS

11.1 SUMMARY

11.1.1 Monitoring HLA-Specific Antibodies

This thesis has highlighted some of the pitfalls involved in the use of microbead assays to monitor the changes in HLA-specific antibody levels. Factors to consider include the density of antigen bound to the bead, the composition of the antibodies within the sera, and also the relevant concentrations of these antibodies. The identification of the high dose hook effect (HDHE) as a cause of false low readings in the microbead assay is of importance when determining relative changes in antibody level through the course of a transplant. Data shown in chapter 9 demonstrated that steric hindrance is the likely cause of the HDHE, as antibodies competing for binding sites in close proximity to each other prevent successful binding. It is important to note that the HDHE is only one of a multitude of factors that may lead to false low readings. Assay interference due to IgM binding, or the presence of the prozone effect must also be considered when interpreting microbead data.

11.1.2 Epitope Determination by Soluble Inhibition (sInh).

Defining the epitope specific nature of anti-HLA antibody binding using soluble inhibition provided a valuable insight into the hidden levels of complexity associated with the HLA-specific antibodies found in patient sera. The observation that anti-A2 reactivity for example is often a mixture of 2,3 or 4 different antibodies, demonstrates that antibody formation in response to foreign HLA is a complex process, indeed there is a need to stop thinking of HLA mismatching in terms of a mismatched antigen but rather in terms of potential mismatched epitopes.

The large panel of sHLA specificities available for this project allowed the identification of a range of HLA class I epitope specificities to be established and also provided the early proof of principle data to allow us to proceed with attempts to design a HLA-specific antibody depletion column with confidence. Indeed this increased understanding of the epitope specific nature of anti-HLA binding was used in the clinical setting to better understand the changes associated with the combined kidney-liver transplant case study outline in chapter 11. Without the knowledge of the epitopes the antibody data would have been much more difficult to interpret, and we could not have fully understood the extraordinary dynamics of antibody absorption displayed by the donor liver.

11.1.3 Quantification of HLA-Specific Antibodies.

There had been no previous reports of successful quantification of HLA-specific antibodies. Recently, there has been much attention on the use of MFI values to monitor changes in antibody levels but these do not give insight into the typical range of concentration at which these antibodies circulate. Using HLA-specific monoclonal antibodies which were then purified and accurately quantified, we were able to construct standard curves using single antigen beads and for the first time it was possible to estimate the typical circulating concentration ranges of these antibodies.

In sensitised waiting list patients circulating HLA-specific antibodies are typically found at concentrations of up to 50μg/ml, and at times of rapid antibody production, such as during an episode of acute antibody mediated rejection, can rise to four or five times this concentration. This range of concentration typically means that HLA-specific antibodies will make up less than 1% of circulating IgG.

Due to the fact that HLA-specific antibodies typically share epitopes it is important to be able to distinguish the exact epitope specificity of an antibody before attempting to quantify using standard curves. Therefore the sInh process to determine epitope specificity went hand in hand with attempts to determine the serum concentration of these antibodies. Without epitope level characterisation it is impossible to accurately quantify these antibodies, as many MFI values would be a composite of more than one antibody, each one at a different concentration.

11.1.4 HLA-Specific IgG Subclass Distribution and Clinical Relevance.

Chapter 6 provided the first full description of HLA-specific IgG subclasses. The data also correlated the subclass distribution with the likely mode of sensitisation. Antibodies stimulated by pregnancy and previous transplant mismatched antigen showed the greatest degree of class switching, with a large proportion of responses class switching all the way though to IgG4.

It was also of note that IgG4 characterised nearly all HLA DQ-specific antibodies stimulated by graft rejection ($p=0.006$). Such widely varying IgG subclass heterogeneity is likely to be due to underlying immunological processes dependent on the route of sensitisation. This diversity, which implies functional variation, may help explain why HLA-specific antibodies are an obstacle to transplantation in some circumstances but not others. The subclass association with rejection has potential as a biomarker for chronic rejection.

Therefore chapter 7 investigated the influence of IgG subclasses in HLAi transplantation. Recent studies which analysed the pre-transplant samples in an incompatible transplant cohort suggested that there is little clinical benefit to measuring IgG subclass levels pre-transplant [158]. However, a more detailed analysis here

showed that pre-transplant class II IgG4 DSA levels were associated with rejection in the early post-transplant period. In addition, post-transplant IgG4 levels displayed unique kinetics compared to the other subclasses (figure 7.4).

11.1.5 Development of HLA Columns

Current technologies to remove antibodies prior to transplantation do not provide the specificity and often remove antibodies to other targets which may compromise patient immunity. Therefore the aim was to develop a device in the form of a column containing HLA antigen bound onto a substrate, through which the patient's plasma will be passed and the HLA antibodies removed.

The soluble HLA (sHLA) proteins used had been previously tested and characterised and used to define epitope specificities. The ability of HLA columns to deplete anti-HLA reactivity exceeded initial expectations, with a 1mg HLA column able to deplete over 90% of specific reactivity from 5ml of patient serum or plasma (figure 8.3).

The project then described pilot studies using clinical scale HLA-A2 and HLA-DR11 column containing around 50-55mg of immobilised HLA. These columns were also highly effective in depleting HLA-specific reactivity, this time from sample volumes in excess of 2 litres in a single treatment. These proof of principle studies provide early promise that a fully licensed therapeutic device may in time be developed. There is still much to be considered, for example a single HLA specificity per column would be of limited clinical benefit in a patient with multiple DSA specificities. The strategy for such patients would have to be considered carefully; multiple columns with different specificities could be used, or alternatively a single column containing a panel

of carefully selected HLA specificities designed to cover as many common epitopes as possible.

The other great benefit that HLA columns provided was the unique ability to isolate HLA-specific antibody from patient material and enrich these antibodies for use in further research. From clinical scale depletion and elution cycles it was possible to isolate up to 20mg of HLA-specific antibody. This valuable resource has many potential applications in both research and clinical service provision. HLA-specific antibodies, fractionated and carefully standardised could serve as an excellent standard reagent for antibody screening, identification, and crossmatch procedures.

An example of a research application for fractionated HLA-specific antibodies was described in chapter 9. The use of carefully produced antibody preparations specific for a single HLA epitope allowed the thorough examination of the factors which determine cytotoxicity. This analysis was able to identify that these antibodies act in a synergistic manner to more effectively cross-link C1q and bring about a cytotoxic reaction. It was also demonstrated that this synergy is also dependant upon the physical location of the epitopes on the HLA moleucle with the added layer of complexity being that if two epitopes are too closely situated together then cytotoxicity can be reduced due to a steric hindrance effect. This same situation occurred on the surface of the single antigen bead, thus serving to further highlight the importance of being able to effectively identify the epitope specific nature of HLA-specific antibody binding. The correct interpretation of both microbead analysis, and cellular crossmatching is reliant upon the identification of epitope binding specificity.

11.2 FUTURE DIRECTIONS

11.2.1 Defining HLA-Specific Antibody Affinity

The work done in this thesis to characterise the antibodies that give rise to the complications within antibody incompatible renal transplantation will provide important insights into the mechanisms underpinning donor organ rejection. However, one area of antibody incompatible transplantation that remains under-represented is that of understanding the biochemical affinity of antibody-antigen interactions. As described in chapter 1 many recipients are able to accommodate organs in spite of their producing antibodies against donor antigens, whilst others suffer severe rejection episodes. One hypothesis relating to this states that the production of donor specific antibodies with high affinity for incompatibility antigens leads to the generation of immune complexes of greater stability within the donor kidney.

Surface plasmon resonance (SPR) is a powerful biophysical technique that facilitates the determination of biochemical kinetic parameters such as association and dissociation constants. Measurements are made in real time and can be supported within temperature ranges between 4-40°C. Sample quantity requirements operate very comfortably within the microgram range. Broader analyses can be carried out using this technique including equilibrium kinetics, concentration measurements and thermodynamics.

The University of Warwick has recently made significant investments in the field of surface plasmon resonance with the purchase of a Biorad XPR36 biosensor, installed at the Walsgrave Campus site of site of the Clinical Sciences Research Institute. At present it is one of only two such instruments installed with a UK

university (the other in Oxford) and is the only one of its kind in the UK to be installed proximal to a major hospital and renal transplantation site. The instrument utilises a removable chip system wherein selected ligands are immobilised on a nano-gold surface via a variety of conjugation chemistries including amine coupling, streptavidin-biotin and thiol coupling. The microfluidic flow-cell set-up can accommodate up to 36 simultaneous sensorgrams involving up to 6 immobilised ligands on the chip and 6 fluid-phase analytes in the flow scheme. The XPR36 instrument is ideally suited to the kinetic analysis of donor-specific antibodies, with the capacity to provide large datasets efficiently from modest amounts of patient plasma.

We have begun work to characterise the binding of HLA-specific antibodies using sHLA coupled to the chip surface. Early validation experiments have been performed using HLA-specific antibodies purified from the HLA protein columns and human HLA-specific monoclonal antibodies kindly donated by Dr Arend Mulder at the University of Leiden, the Netherlands. Figure 11.1 shows the example of a HLA-specific monoclonal antibody binding to a sHLA-A2 protein immobilised to the SPR chip.

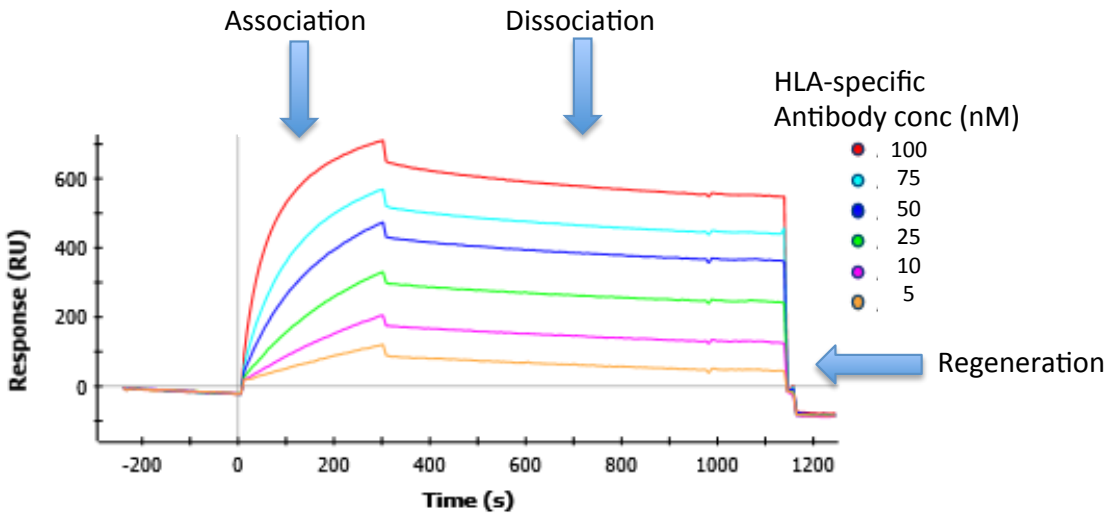


Figure 11.1: SPR sensorgram showing binding of fractionated HLA-specific antibody. The response curve in the association phase can provide an 'on-rate', with the rate of dissociation showing the 'off-rate' of the antibody. The chip surface can then be regeneration using alkaline elution.

The binding of HLA-specific antibodies is expected to be highly complex in patient sera, with antibodies existing as a polyclonal mixture of antibodies albeit exhibiting the same binding specificity. This assumption is based upon work carried out by our research group on ABO antibodies, which demonstrated the complex mix of antibodies present within sera. With this in mind we have entered into a fruitful collaboration with colleagues in the Engineering Department at the University of Warwick, and have published work describing the modelling of SPR data to define ABO and HLA binding affinities [190, 191].

Work will continue to define the assay and a clinical study is planned to monitor the changes in HLA-specific antibody affinity over time and to correlate this to graft outcome.

Chapter 12

ASSOCIATED PEER-REVIEWED PUBLICATIONS

Lowe, D., R. Higgins, et al. (2013). "Significant IgG subclass heterogeneity in HLA-specific antibodies: Implications for pathogenicity, prognosis, and the rejection response." Hum Immunol.

Abstract: IgG subclasses differ in their ability to fix complement and bind Fc receptors. This study describes a detailed analysis of the distribution of HLA-specific IgG subclasses in order to define how this varies in sensitised waiting-list patients. We found significant variation in the level, presence and combinations of each HLA-specific IgG subclass between and within individuals and this is influenced by the type of sensitising event. Graft failure in particular provokes higher levels of IgG1 (vs transfusion, p=0.071 and pregnancy, p=0.042), IgG2 (vs transfusion, p=0.001 and pregnancy, p=0.016), and IgG4 (vs transfusion, p=0.052). Both graft failure and pregnancy tend to stimulate multiple IgG subclass responses against HLA, whereas transfusion stimulated antibodies are dominated by responses limited to IgG1 (p=0.033) and have a low incidence of IgG4 (p=0.046). In marked contrast, IgG4 characterised nearly all HLA DQ-specific antibodies stimulated by graft rejection (p=0.006). Such widely varying IgG subclass heterogeneity is likely to be due to underlying immunological processes dependent on the route of sensitisation. This diversity, which implies functional variation, may help explain why HLA-specific antibodies are an obstacle to transplantation in some circumstances but not others. The subclass association with rejection has potential as a biomarker for chronic rejection.

Higgins, R., D. Lowe, et al. (2011). "Human leukocyte antigen antibody-incompatible renal transplantation: excellent medium-term outcomes with negative cytotoxic crossmatch." Transplantation 92(8): 900-906.

Abstract: Human leukocyte antigen (HLA) antibody-incompatible renal transplantation has been increasingly performed since 2000 but with few data on the medium-term outcomes. METHODS: Between 2003 and 2011, 84 patients received renal transplants with a pretreatment donor-specific antibody (DSA) level of more than 500 in a microbead assay. Seventeen patients had positive complement-dependent cytotoxic (CDC) crossmatch (XM), 44 had negative CDC XM and positive flow cytometric XM, and 23 had DSA detectable by microbead only. We also reviewed 28 patients with HLA antibodies but no DSA at transplant. DSAs were removed with plasmapheresis pretransplant, and patients did not routinely receive antithymocyte globulin posttransplant. RESULTS: Mean follow-up posttransplantation was 39.6 (range 2-91) months. Patient survival after the first year was 93.8%. Death-censored graft survival at 1, 3, and 5 years was 97.5%, 94.2%, and 80.4%, respectively, in all DSA+ve patients, worse at 5 years in the CDC+ve than in the CDC-ve/DSA+ve group at 45.6% and 88.6%, respectively (P<0.03). Five-year graft survival in the DSA-ve group was 82.1%. Rejection occurred in 53.1% of DSA+ve patients in the first year compared with 22% in the DSA-ve patients (P<0.003). CONCLUSIONS: HLA antibody-incompatible renal transplantation had a high success rate if the CDC XM was negative. Further work is required to predict which CDC+ve XM grafts will be successful and to treat slowly progressive graft damage because of DSA in the first few years after transplantation.

Higgins, R., D. Lowe, et al. (2010). "Double filtration plasmapheresis in antibody-incompatible kidney transplantation." Ther Apher Dial 14(4): 392-399.

Abstract: Double filtration plasmapheresis (DFPP) was used in preference to plasma exchange in our program of antibody-incompatible transplantation, to treat higher volumes of plasma. Forty-two patients had 259 sessions of DFPP, 201 pre-transplant and 58 post-transplant. At the first treatment session, the mean plasma volume treated was 3.81 L (range 3-6 L), 55.5 mL/kg (range 36.2-83.6 mL/kg). Serum IgG fell by mean 59.4% (SD 10.2%), and IgM by 69.3% (SD 16.1%). Nine patients did not require increases in plasma volumes treated, and six did not tolerate higher plasma volumes. In the remaining patients, the mean maximum plasma volume treated pre-transplant was 6.67 L (range 4-15 L), 96.1 mL/kg (range 60.2-208.9 mL/kg). The complement dependent cytotoxic crossmatch was positive in 14 cases pre-treatment, and remained positive in six (42.8%) cases. The flow cytometric crossmatch was positive in 29 cases pre-treatment, and in 21 (72.4%) after DFPP. Post-transplant, DFPP was ineffective at reducing donor specific antibody levels during periods of rapid donor specific antibody synthesis. Post-transplant, the one year graft survival rate was 94%, although there was a high rate of early rejection. In summary, DFPP enabled the treatment of plasma volumes that were almost double those that would have been feasible with plasma exchange. Despite this, most patients were transplanted with a positive crossmatch, and DFPP post-transplant was unable to control rising antibody levels.

Higgins, R., D. Lowe, et al. (2009). "Rises and falls in donor-specific and third-party HLA antibody levels after antibody incompatible transplantation." Transplantation 87(6): 882-888.

Abstract. After human leukocyte antigen (HLA) antibody-incompatible transplantation, donor specific and third party HLA antibodies may be found, and their levels fall in a donor-specific manner during the first month. However, these changes have not been previously described in detail. METHODS: Donor-specific HLA antibody (DSA) and third-party HLA antibody (TPA) levels were measured using the microbead method in 44 presensitized patients who had renal transplantation. RESULTS: DSA+TPA fell in the first 4 days after transplantation, and greater falls in DSA indicated absorption by the graft. This occurred for class I (57.8% fall compared with 20.2% for TPA, P<0.0005), HLA DR (63.0% vs. 24.3%, P<0.0004), and for HLA DP/DQ/DRB3-4 (34% vs. 17.5%, P=0.014). Peak DSA levels occurred at a mean of 13 days posttransplant, and they were higher than pretreatment in 25 (57%) patients and lower in 19 (43%) patients (P=ns). The risk of rejection was associated with peak DSA levels; 15 of 25 (60%) patients with DSA at median fluorescence intensity (MFI) more than 7000U experienced rejection, compared with 4 of 7 (57%) patients with peak DSA MFI 2000 to 7000U, and 2 of 12 (17%) patients with peak DSA MFI less than 2000U (P<0.02). DSA levels subsequently fell in a donor specific manner compared to TPA. CONCLUSION: DSA levels may change markedly in the first month after antibody incompatible transplantation, and the risk of rejection was associated with higher pretreatment and peak levels.

Higgins, R., M. Hathaway, D. Lowe et al. (2007). "Blood levels of donor-specific human leukocyte antigen antibodies after renal transplantation: resolution of rejection in the presence of circulating donor-specific antibody." Transplantation 84(7): 876-884.

Abstract: Accommodation to antibody is an important mechanism in successful ABO-incompatible transplantation, but its importance in human leukocyte antigen (HLA) antibody-incompatible transplantation is less clear, as sensitive techniques facilitating daily measurement of donor-specific HLA antibodies (DSAs) have only recently been developed. METHODS: We report 24 patients who had HLA antibody-incompatible kidney transplantation (21 living donors, 3 deceased), 21 of whom had pretransplant plasmapheresis. Eight had positive complement-dependent cytotoxic (CDC) crossmatch (XM) pretransplant plasmapheresis, nine had positive flow cytometric (FC) XM, and seven had DSA detectable by microbead analysis only. After transplant, DSA levels were monitored closely with microbead assays. RESULTS: Rejection occurred in five of eight (62.5%) CDC-positive cases, in three of nine (33%) FC-positive cases, and in two of seven (29%) of microbead-only cases at a median of 6.5 days after transplantation. Resolution occurred at a median of 15 days after transplantation, in 8 of 10 cases when the microbead level of DSA had median fluorescence intensity (MFI) >2000 U, in 6 of 10 when the microbead MFI >4000 U. In 8 of 10 cases, the microbead MFI at the time of resolution was greater than at the onset. DSA did not always cause clinical rejection. In five cases with a posttransplant DSA peaking at MFI >2000 U on microbead assay, rejection did not occur. CONCLUSION: These data suggest that the dominant method of successful transplantation was function of the transplant in the presence of circulating DSA, and they also define the period during which this occurred.

Chapter 13:

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